Conformation States of Concanavalin A: Kinetics of Transitions Induced by Interaction with Mn^{2+} and Ca^{2+} Ions†

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ABSTRACT: Using measurements of the magnetic-field dependence of the nuclear magnetic relaxation rate ($1/T_1$) of solvent water protons over a wide range of field values (corresponding to proton Larmor frequencies from 0.01 to 50 MHz), we have investigated the interaction of Mn^{2+} and Ca^{2+} ions with concanavalin A (Con A) over the pH range 5.3 to 6.4, at 5 and 25 °C. Particular attention was given to time-dependent effects that occur upon addition or removal of metals. Limited amounts of Mn^{2+} added to solutions of apo-Con A bind at S1 (the usual "transition-metal" site) to form a binary complex characterized by a large and pH-dependent dissociation constant, rapid exchange of Mn^{2+} ions with solvent, and a relatively large and pH-independent contribution to the proton relaxation rate. With S1 occupied, Ca^{2+} ions can bind at S2 (the usual "calcium-binding" site) to form a metastable ternary complex characterized by a relatively large and pH-dependent dissociation constant for Ca^{2+} ions, rapid exchange of Ca^{2+} ions with solvent, and a relatively low and pH-independent contribution to the proton relaxation rate. We find that this metastable ternary complex undergoes a first-order transition to a stable ternary complex, with a pH-independent time constant of 17 ± 1 min at 5 °C and an activation energy of 22 kcal M^{-1}. This stable ternary complex has the same relaxation contribution as the initial metastable complex, but differs in that the dissociation constant of Ca^{2+} is very low; the off-rate of both metals is of the order of days at 25 °C. Saccharide binding and agglutination studies are generally done with this form of Con A. We have also found that, in the absence of Ca^{2+}, Mn^{2+} can bind at S2 as well as at S1 (S2 was previously thought to bind only Ca^{2+} and Cd^{2+}) to form a metastable ternary complex which, like the metastable Mn^{2+}–Ca^{2+}–Con A complex, undergoes a transition to a stable state, but with a time constant that is much larger than for the Ca^{2+}-containing ternary complex. In contrast to the stable Ca^{2+}–Mn^{2+}–Con A complex, the stable Mn^{2+}–Con A ternary complex has a rather large dissociation constant, and the bound Mn^{2+} ions are in rapid equilibrium with solvent. The Mn^{2+} ions can be removed rapidly by the addition of ethylenediaminetetraacetic acid to produce apo-Con A in a metastable state that has different metal-binding properties than apo-Con A prepared by acid demetallization of the protein. Metals reintroduced to this metastable form of apo-Con A produce the stable ternary complexes with no observable time-dependent effects. This metastable form of apo-Con A reverts to its initial state after several days at 25 °C. We have fit the kinetic and thermodynamic data for the interaction of Mn^{2+} and Ca^{2+} ions with Con A with a model that postulates two conformation states for Con A that differ only slightly in their ground-state free energies, and are separated by an energy barrier of 22 kcal M^{-1}. The conformation with the lower free energy is determined by the presence or absence of a metal ion at S2. The height of the energy barrier suggests that a cis–trans isomerization of a proline amide bond distinguishes the two conformations, implying that the difference between the conformations is in the secondary rather than the tertiary structure of the protein.

Concanavalin A, a metallo-protein isolated from the jack bean (*Canavalia ensiformis*), is one of a number of plant lectins. These proteins agglutinate cells in suspension with a selectivity that relates to the ability of lectins to bind to specific saccharides on cell surfaces (cf. Sharon and Lis, 1972; Lis and Sharon, 1973, for reviews). Interest in Con A[1] in particular arises from additional biological effects associated with the interaction of this protein with cell-surface saccharides; e.g., Con A is reported to agglutinate oncogenic cells preferentially (Inbar and Sachs, 1969), to restore normal growth behavior to tissue cultures of transformed fibroblasts (Burger and Noonan, 1970), and to alter the expression of a cell-surface enzyme (Painter and White, 1976).

Below pH of about 6, Con A is a dimer of two essentially identical monomers of molecular weight 27 000, and above pH of about 7 it is a tetramer (McKenzie et al., 1972). For some time it has been known that the saccharide-binding and agglutination properties of Con A require the presence of divalent cations (Yariv et al., 1968; Kalb and Levitzki, 1968; Agrawal and Goldstein, 1968; Inbar and Sachs, 1969). There are two cation binding sites per monomer: a site S1 that binds a variety of divalent transition-metal ions, including Mn^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+}, and a site S2 that binds Ca^{2+} and Cd^{2+} (Kalb and Levitzki, 1968; Shoham et al., 1973). S2 is not formed until S1 is occupied, and occupation of both sites is believed necessary for saccharide binding (Agrawal and Goldstein, 1968) and agglutination activity (Inbar and Sachs, 1969).

It is known from x-ray crystallographic studies that S1 and S2 are accessible to solvent and that metal ions in S1 and S2 are liganded to the same carboxyl groups of two aspartic acid

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[1] Abbreviations used are: Con A, concanavalin A with unspecified metal content; P, MP, MMP, and CMP, apo-Con A, the Mn^{2+}–Con A binary complex, the Mn^{2+}–Con A ternary complex, and the Ca^{2+}–Mn^{2+}–Con A ternary complex, respectively, of one particular conformation (called "unlocked") of Con A; PL, MPL, MMPL, and CMPL, the analogous forms of another conformation (called "locked") of Con A; MPE and CMPE, the thermal equilibrium states of samples of apo-Con A to which Mn^{2+}, and subsequently Ca^{2+}, respectively, have been added; [PS], [MPS], and [MMPS], the sums of the concentrations of the respective unlocked and locked complexes; M and C, Mn^{2+} and Ca^{2+} ions, respectively, free in solution; EDTA, ethylenediaminetetraacetic acid.

residues (Weinzierl and Kalb, 1971; Edelman et al., 1972; Hardman and Ainsworth, 1972), and from nuclear magnetic resonance (Brewer et al., 1973a,b; Villafranca and Viola, 1974; Alter and Magnuson, 1974) and x-ray studies (Hardman and Ainsworth, 1976; Becker et al., 1976) that the saccharide binding site is located about 12 Å from S1.

Recent data indicate that there are additional sites that bind metals. Pb^{2+} and several rare earth ions have been shown to bind elsewhere than at S1 and S2 (Sherry and Cottam, 1973; Becker et al., 1975; Hardman and Goldstein, 1977), and Richardson and Behnke (1976) reported that Ca^{2+} can also bind at sites other than S2.

Several experiments indicate that the addition of Ca^{2+} to Con A alters the properties of the metal ion at S1: the electron-spin resonance spectrum of bound Mn^{2+} is altered (Nicolau et al., 1969), the spin-lattice (longitudinal) relaxation rate ($1/T_1$) of solvent protons is decreased due to a decrease in the off-rate of an exchanging water ligand of the Mn^{2+} (Meirovitch and Kalb, 1972; Barber and Carver, 1974; Sherry and Cottam, 1973), the circular dichroism spectrum of the protein in the near ultraviolet is changed (Barber and Carver, 1974), and the optical spectrum of bound Co^{2+} is altered (Richardson and Behnke, 1976). These effects could result from a direct interaction between the two metal ions per monomer, since they are bound to common amino acid residues. However, several experiments suggest that the effects associated with binding of Ca^{2+} are indicative of a conformation change of the protein itself. Weinzierl and Kalb (1971) observed an alteration of the space group of a single crystal of Mn^{2+}-Con A upon addition of Ca^{2+}, and several workers (Barber and Carver, 1973; Grimaldi and Sykes, 1975; and Richardson and Behnke, 1976) have reported slow time-dependent changes in either the properties of the ion at S1 or the activity of the protein upon addition of Ca^{2+}.

We have studied the interaction of Mn^{2+} and Ca^{2+} ions with Con A at 5 and 25 °C over the pH range 5.3 to 6.4, with particular attention to time-dependent effects, using a nuclear magnetic relaxation technique that is especially suited to investigating the interactions of Mn^{2+} ions with macromolecules (see below). We find that, indeed, binding of Ca^{2+} to the binary complex of Mn^{2+}-Con A initiates a conformation change in the protein that manifests itself as a time-dependent uptake of metal ions from solution. We have also discovered that, in the absence of Ca^{2+}, excess Mn^{2+} can bind at S2 in addition to S1 and induce similar time-dependent changes in the properties of the protein. The effects of Mn^{2+} ions binding at S2 are qualitatively similar to those induced by Ca^{2+} binding; however, the binding of Mn^{2+} is weaker, the changes are not so dramatic, and the time course of the changes typically extends over many hours at room temperature after Mn^{2+} addition, compared to a few minutes after Ca^{2+} addition. Nonetheless, it is clear that S2 can bind Mn^{2+}, contrary to what has generally been believed.

From measurements of the kinetics of the change in nuclear magnetic relaxation rates of solutions of Mn^{2+}-Con A induced by addition of Ca^{2+}, we have developed a simple model that describes a wide range of quantitatively different kinetic data. The model proposes that Con A can exist in two conformations that are separated by a high-energy barrier; occupation of S2 determines which conformer has the lower free energy. We have derived values for the rate constant that describes the change of conformation, and for its activation energy. Based partly on the values of these parameters, we propose that the conformation change is in the secondary structure of the protein, and involves a cis–trans isomerization of a proline amide bond. By judicious addition and removal of metal ions, and by taking advantage of the long time for interconversion of the two conformers at low temperatures, we have produced both apoprotein and the binary Mn^{2+}-Con A complex in a metastable conformation. We have also obtained values for several of the dissociation constants of Mn^{2+} and Ca^{2+} from the different metal ion-Con A complexes that can be prepared.

We have previously studied the stable ternary Ca^{2+}-Mn^{2+}-Con A complex by examining its effects on the $1/T_1$ of solvent water protons as a function of magnetic field at several temperatures (Koenig et al., 1973). The present studies are an extension of these procedures and techniques; we call the method magnetic relaxation dispersion, and a graph of the variation of $1/T_1$ with magnetic field a dispersion curve. Mn^{2+} ions are particularly effective in relaxing solvent protons (so long as these ions are accessible to solvent), and the observed dispersion curves are the sums of unique dispersion contributions from Mn^{2+} ions in different types of sites. In particular, the Mn^{2+} aquoion dispersion varies in the field region below about 150 Oe, corresponding to proton Larmor frequencies below about 0.6 MHz,[2] whereas we have found no form of Mn^{2+} bound to protein with a dispersion that varies with field in this region. On the other hand, bound Mn^{2+} ions typically have a peak in their dispersion above about 10 MHz, where the aquoion contributes very little. Thus, by measuring the proton relaxation dispersion of a solution of protein containing Mn^{2+} ions over a wide range of magnetic fields, including very low values, we can obtain the dispersion contributions of the Mn^{2+} aquoion and various forms of Mn^{2+}-containing protein. From such data, we can characterize the various Mn^{2+}-protein complexes and determine their concentrations. This capability of magnetic relaxation dispersion measurements makes them uniquely suited for the present study, and for the study of interactions of Mn^{2+} ions with macromolecules in general, provided one can obtain data at very low magnetic fields.

The interpretation of the data in terms of the different types and concentrations of Mn^{2+} sites does not require a detailed understanding of the mechanisms by which Mn^{2+} ions relax solvent protons, but only the empirical profile of the dispersion contribution for each type of Mn^{2+} site. In the present paper, we use fits of the dispersion theory to data only to determine the off-rate of water ligands of the bound Mn^{2+} ions. There are, in fact, basic questions regarding the mechanism of relaxation of solvent protons by Mn^{2+} ions bound to protein, and the interpretation of relaxation dispersion in terms of fundamental parameters (Koenig et al., 1971; Koenig and Epstein, 1975); the relevance of the present data to questions regarding the theory of relaxation will be considered in a separate publication.

The long-range goal of these studies is to understand the factors that influence the specificity of interactions between Con A (and lectins in general) and saccharides. The present work is restricted to considerations of the different conformers of Con A and their respective interactions with Mn^{2+} and Ca^{2+}. The interactions of the several forms of metal-Con A complexes with saccharides, and the accompanying time-dependent effects associated with slow conformational changes in the protein molecules, will be considered in a separate publication.

Materials and Methods

Sample Preparation. To prepare apo-Con A, native Con A (Miles-Yeda or Sigma) was demetallized by lowering the pH

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

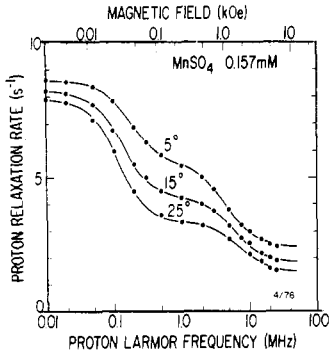

FIGURE 1: Solvent proton relaxation dispersion of a solution of Mn^{2+} aquoions at three temperatures. The sample was a solution of 0.157 mM $MnSO_4$ in 0.1 M potassium acetate, 0.9 M potassium chloride buffer, pH 5.6.

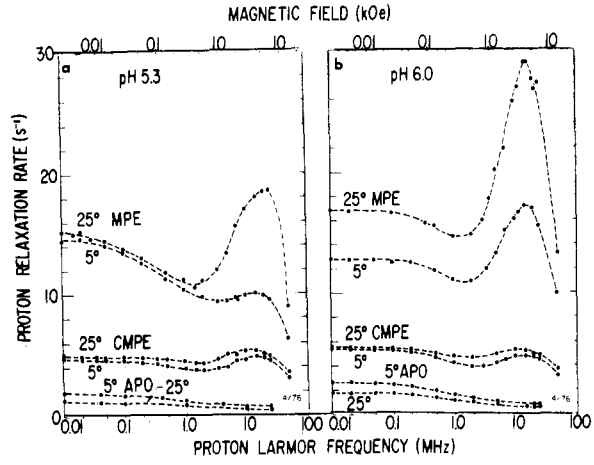

FIGURE 2: Solvent proton relaxation dispersion data for two of four samples of apo-Con A to which Mn^{2+} ions and, subsequently, saturating concentrations of Ca^{2+} ions were added. Each sample is at a different pH, and data are presented for two temperatures at each pH. Dispersion data are shown for solutions of the apoprotein, the Mn^{2+}-Con A complexes (MPE), and the Ca^{2+}-Mn^{2+}-Con A complexes (CMPE). The suffix "E" indicates that the data were taken after all drifting of the relaxation rates had stopped, and the samples had reached equilibrium. (a) pH 5.3, total Mn^{2+} ion concentration $[M_T]$ = 0.33 mM, total protein monomer concentration $[P_T]$ = 0.74 mM, total Ca^{2+} ion concentration $[C_T]$ = 1.67 mM; (b) pH 6.0, $[M_T]$ = 0.31 mM, $[P_T]$ = 1.26 mM, $[Ca_T]$ = 0.67 mM. The buffer in all cases was, as in Figure 1, 0.1 M potassium acetate, 0.9 M potassium chloride. The smooth curves through the data points are drawn solely to improve the legibility of the data.

of a solution of 1.0 g of protein in 30 mL of distilled and deionized water to 1.2 by addition of 1 N HCl, while stirring at 25 °C. After 45 min, the solution was transferred to dialysis bags (which had been boiled in a 1 mM EDTA solution), and dialyzed against two successive 6-L volumes of 1 mM EDTA in distilled water at 4 °C. The solution was further dialyzed against three successive 1-L volumes of the final buffer (0.1 M potassium acetate, 0.9 M KCl) at the appropriate pH at 4 °C. (High ionic strength buffer must be used at this point to ensure complete removal of EDTA from the protein; we found that final dialysis against a low ionic strength buffer results in residual EDTA binding to apo-Con A. Subsequent addition of metals to the apoprotein scavenges the residual EDTA and produces anomalous relaxation results. Fee (1973) has reported similar problems in preparations of superoxide dismutase.) The resulting mixture was centrifuged at 10 000 rpm in an SS-34 Sorval rotor for 15 min. The supernatant yields approximately 25 mg/mL of apo-Con A; this material was used directly.

The final protein concentration was determined spectrophotometrically at pH 5.6 using an absorbance $A_{280nm}^{1\%,1cm}$ = 12.4 (Yariv et al., 1968). Stock solutions of 0.1 M $MnCl_2$, $MnSO_4$, and $CaCl_2$ were standardized by EDTA titration. Microliter amounts were added directly to apo-Con A solutions for the experiments reported here so that the total metal content of each sample was known. Mn^{2+} and Ca^{2+} concentrations in some protein samples were checked by atomic absorption analysis, for which reference samples were prepared in the buffer used for the protein sample in order to correct for background effects introduced by buffer ions. Nonetheless, we find that significant systematic errors can occur in metal determination by atomic absorption analysis, perhaps due to the presence of protein, and therefore these results have only been used as a qualitative guide.

Relaxation Measurements. Relaxation measurements were made by the field cycling method previously described (Koenig and Schillinger, 1969; Hallenga and Koenig, 1976). Sample volume was routinely 0.6 mL. Reproducibility of the data for a given sample was generally better than ±2%. The time required for a typical measurement of $1/T_1$ was about 4 min.

Experimental Results

The Mn^{2+} Aquoion. Figure 1 shows proton relaxation dispersion data for 0.157 mM $MnSO_4$ in pH 5.6 buffer at three temperatures. This Mn^{2+} concentration is typical of that used in the protein-containing samples. The relaxation rates are independent of pH over a wider range than is our present concern, but at low fields are about 20% less than for distilled water solutions of the same Mn^{2+} concentration. (We find the rates vary with the type of buffer as well; these details will be considered elsewhere.) The solid lines through the data points are the results of a least-squares fit of the well-established theory of proton relaxation by Mn^{2+} aquoions (cf. Koenig et al., 1971; or Hertz, 1973, for details); the values of the theoretical parameters so derived are used to compute the contribution of free Mn^{2+} to the relaxation rates in all protein-containing samples. The concentration of free Mn^{2+} in each case is determined by comparing the difference in relaxation rates at 0.02 and 0.5 MHz with the differences in Figure 1 (after a small correction due to apoprotein, as will be clarified), using the facts that the relaxation contribution of the aquoion is additive, and linear in Mn^{2+} concentration.

Mn^{2+}-Con A and Ca^{2+}-Mn^{2+}-Con A Equilibrium Complexes. Figure 2a,b contrasts the relaxation dispersions at 5 and 25 °C for samples at pH 5.3 and 6.0. (Analogous data were taken at pH 5.6 and 6.4.) In each case, the dispersions of solutions of apo-Con A, Mn^{2+}-Con A, and Ca^{2+}-Mn^{2+}-Con A are shown. The apoprotein was kept at room temperature for about 1 week after its preparation before the first addition of metal, since the relaxation rates of samples freshly prepared or stored at low temperatures drift substantially upon addition of Mn^{2+}. Even so, it may be a few hours before the Mn^{2+}-protein samples stabilize, but the drift in relaxation rate is typically less than 10%. For the data in the figures, the total Mn^{2+} added is much less than the concentration of monomer, whereas the Ca^{2+} concentration is in excess of that of the monomer. Ca^{2+} was added at 5 °C, and the time course of the relaxation rate was followed (see below). The amount of Ca^{2+} added was chosen so that the relaxation rates of the samples would stabilize within a few hours. The dispersion data in Figure 2a,b were taken about 24 h after the addition of Ca^{2+}, during which time the samples were kept at 25 °C. We will refer to samples containing only Mn^{2+} and protein, with data taken after waiting for relaxation rates to stabilize, as MPE

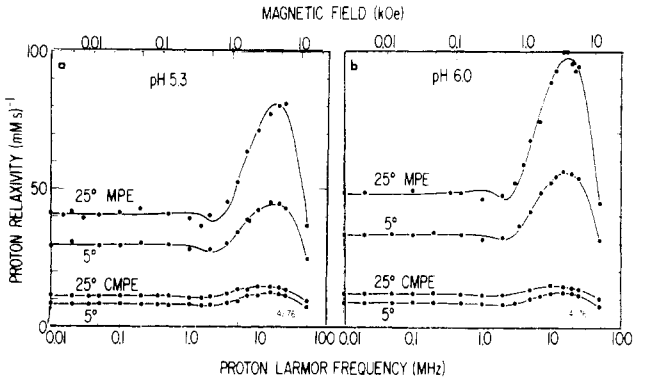

FIGURE 3: Solvent water proton relaxivity, the relaxation contribution per millimole of Mn^{2+} complex, for Mn^{2+} ions bound to apoprotein at equilibrium (MPE) and bound in the ternary Ca^{2+}-Mn^{2+}-Con A complex at equilibrium (CMPE), at two temperatures and pH 5.3 and 6.0. The results are derived from the data in Figure 2a,b. The solid lines represent the results of a least-squares fit of the theory of relaxation of solvent protons by paramagnetic ions to the data.

("E" for "equilibrium"); similarly, we will refer to protein samples containing both Mn^{2+} and Ca^{2+}, with data taken after all drifting has stopped, as CMPE.

There is a number of qualitative observations to be made concerning the data (Figure 2a,b). The relaxation rates of the Mn^{2+} complexes in the absence of Ca^{2+} ions are much higher than in the presence of Ca^{2+}, and the relaxation rates of the apoprotein are comparatively small. Assuming that the only contribution to a significant field dependence of the relaxation rate below about 1 MHz is from the Mn^{2+} aquoion, there is no observable free Mn^{2+} in any of the CMPE samples. The binding of Mn^{2+} to apoprotein to form MPE increases significantly with increasing pH, and increases with increasing temperature at the lower pH values. The peak in the relaxation rate in the range 10–20 MHz, which is a feature of bound Mn^{2+}, is much less at 5 than at 25 °C for the MPE samples, and is smaller still for CMPE at each temperature.

The raw data have been reduced to give the relaxivities (relaxation rates per millimole of Mn^{2+}-complex) of MPE and CMPE, and are presented in Figure 3a,b. The procedure used was to subtract the apo-Con A data from the MPE and CMPE data, and then determine the free Mn^{2+} concentration from the difference between the relaxation rates at 0.02 and 0.5 MHz, using the data of Figure 1. The contribution of the free Mn^{2+} was then computed, the appropriate subtractions were made, and the results converted to relaxivities by dividing by the known concentration of bound Mn^{2+}. That only the Mn^{2+} aquoion contributes to the differences in relaxation rates between 0.02 and 0.5 MHz was verified experimentally by dialyzing a sample, for which the free Mn^{2+} concentration was determined as above, against buffer to which this same concentration of free Mn^{2+} was added. If the Mn^{2+} aquoion concentrations in the sample and external to it were not the same, the relaxation rates measured before and after dialysis would have changed; we could detect no change in free Mn^{2+} concentration by this procedure. In computing the relaxivities of the MPE samples, we assumed that all the metal–protein complexes contained a single Mn^{2+} ion per monomer. As will be seen, this is a reasonable first-order procedure, but not exact.

The solid lines in Figure 3a,b result from a fit of theory to the data. The theory and fitting procedures for both MPE and CMPE are as applied previously to CMPE (Koenig et al., 1973), and will not be discussed here. The fits to the data are very good and we use them as an empirical tool for describing the reduced data (cf. the Discussion section).

Several points emerge from the results in Figure 3a,b, and additional results not illustrated. First, the relaxivities of CMPE are independent of pH from pH 5.3 to 6.4. This independence, once demonstrated, is a check on the total Mn^{2+} added, since all Mn^{2+} is taken up in the presence of excess Ca^{2+} and protein. Indeed, in contrast to the weak binding of Mn^{2+} to MPE, both metals in CMPE are so tightly bound that significant amounts of metal can be removed by dialysis (with or without EDTA) only in times of the order of a few days at 25 °C. Second, though the relaxivities of the MPE samples are reasonably independent of pH, it can be seen (particularly, by the height of the peak in the relaxivity at 25 °C) that the relaxivity of the pH 6.0 sample is about 25% greater than that of all the others. The main difference between this sample and the others is that it has about twice the protein content, and therefore the assumption of one bound Mn^{2+} ion per monomer is essentially correct. The origin of the lower relaxivities of the other samples of MPE (which has to do with the binding of two Mn^{2+} ions per monomer) will be examined in detail below.

To summarize the results to this point, we find that there is a form of Con A with high relaxivity, ostensibly with Mn^{2+} bound at S1 to form MPE, and a form with low relaxivity with Mn^{2+} at S1 and Ca^{2+} at S2 to form CMPE. These are equilibrium forms, with the data taken long after the time-dependent relaxation rates have become stationary. Mn^{2+} binds weakly to apo-Con A, and the binding is pH dependent. By contrast, both Mn^{2+} and Ca^{2+} bind tightly to CMPE in our pH range. Finally, there is evidence that, in the absence of Ca^{2+}, Mn^{2+} can bind to a site in addition to S1.

Locking of the Ca^{2+}-Mn^{2+}-Con A Ternary Complexes. It can take a significant time at 5 °C for the relaxation rate to stop decreasing after Ca^{2+} is added to MPE samples. This is illustrated in Figure 4a,b, which shows the time courses of the relaxation rates at 0.02, 0.5, and 15 MHz. The difference in relaxation rates at 0.02 and 0.5 MHz is a measure of the concentration of free Mn^{2+}, as discussed above. The peak in the relaxation rates of the bound Mn^{2+} contribution for both MPE and CMPE is near 15 MHz. The data shown are for samples for which the equilibrium dispersion data are given in Figure 2a,b. Qualitatively, the data are characterized by an immediate initial drop of the relaxation rates upon addition of Ca^{2+} to MPE, and a subsequent monotonic decrease of the relaxation rates at all fields. For the lower pH samples, the decrease in the difference in rates between 0.02 and 0.5 MHz indicates an uptake of free Mn^{2+} with time. (At the higher pH values, the binding is such that there is too little free Mn^{2+} to observe any change.) Since the time-dependent process involves a large increase in the strength of metal binding, we refer to the phenomena as "locking". The solid lines through the data points at each pH are the results of a fit to a model which is discussed in a separate section below. A major point to note is that, at the lower pH, greater total amounts of added Ca^{2+} are required to keep the time course approximately the same.

We have also taken data on locking for two aliquots of a pH 5.6 sample at 5 °C which differ by a factor of two in total Ca^{2+} concentrations; the sample with the greater concentration of Ca^{2+} has the shorter time course. In addition, we have repeated the locking experiments at 25 °C, but the time courses are too rapid to obtain accurate data. Typically, what occurs in hours at 5 °C occurs in minutes at 25 °C.

Though Ca^{2+} binds weakly in the initial ternary complex, it is possible to add sufficient Ca^{2+} to MPE to drive Ca^{2+} onto each monomer containing Mn^{2+}. When this is done, all free Mn^{2+} is taken up by mass action (so long as the total Mn^{2+} is

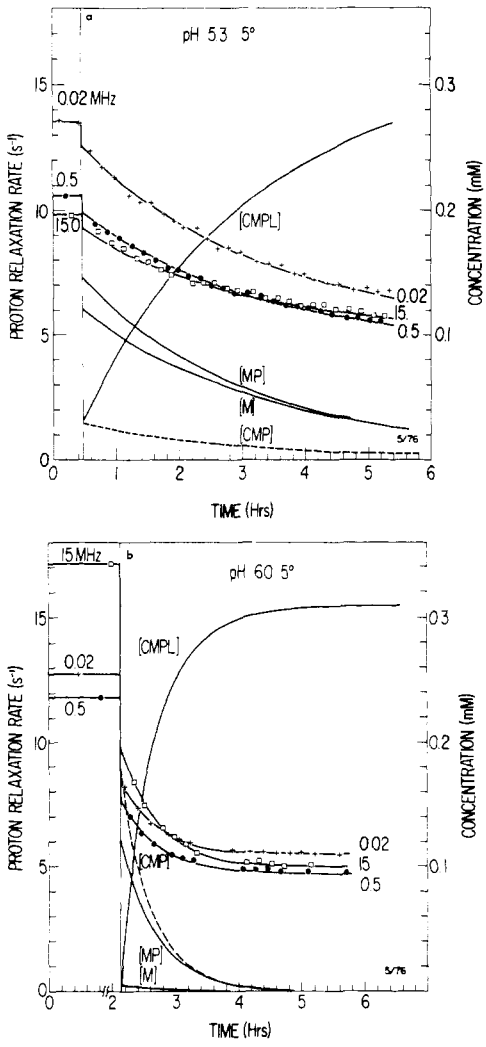

FIGURE 4: Time course of solvent proton relaxation rates for the MPE samples of Figure 2a,b after addition of Ca^{2+} ions at 5 °C, for values of magnetic field corresponding to the proton Larmor frequencies shown. Ca^{2+} was added at the times indicated by the vertical lines near the left of each graph. The lines labeled [M], [MP], [CMP], and [CMPL] are values of the time-dependent concentrations of free Mn^{2+}, the binary Mn^{2+}–Con A complex in the unlocked conformation, and the ternary Ca^{2+}–Mn^{2+}–Con A complex in the unlocked and locked conformations, respectively (see text), computed from a fit of the model, Scheme III, to the data. The solid lines through the data are values of the relaxation rates computed from the model results, using the known values of the relaxivity of each component. The only adjustable parameter was the rate constant for locking, $1/T_L$ (eq 17). Values of K_{MP} and K_{CMP} were computed from the values of the relaxation rates just before and just after Ca^{2+} addition (see text). (a) K_{MP} = 0.43 mM, K_{CMP} = 8.0 mM, T_L = 0.27 h; (b) K_{MP} = 0.039 mM, K_{CMP} = 0.33 mM, T_L = 0.30 h.

less than the monomer concentration), and the relaxation rates become (within a few percent) those for CMPE. Though the relaxation rates do not vary with time, it is straightforward to show experimentally that the locking process is going on. Sufficient EDTA added immediately after addition of Ca^{2+} removes all metal from the protein and from solution, and the resultant relaxation dispersion is that of the Mn^{2+}–EDTA complex. (This complex has a low relaxivity, about 7 $(mM\ s)^{-1}$ at low fields and decreases monotonically above about 5 MHz. These data will not be presented here.) EDTA added a few hours after the addition of Ca^{2+} has little or no immediate effect, and EDTA added at intermediate times has the expected intermediate influence on the relaxation rates.

Equilibrium Titration of Mn^{2+} into Apo-Con A. A sample of apo-Con A was prepared and divided into 25 aliquots. The

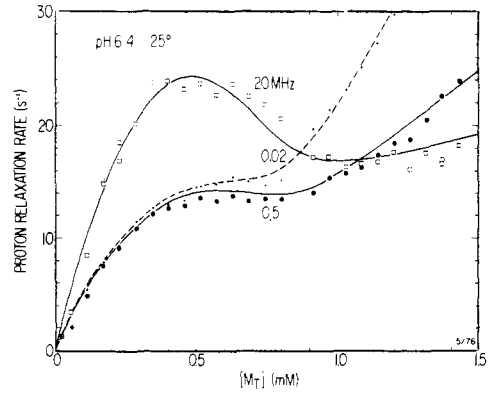

FIGURE 5: Solvent proton relaxation rate at three values of magnetic field, corresponding to proton Larmor frequencies of 0.02, 0.5, and 20 MHz, for samples of apo-Con A with varying concentrations of added Mn^{2+} ions, at 25 °C, in the absence of Ca^{2+} ions. The apo-Con A was 0.40 mM monomer in 0.1 M potassium acetate, 0.9 M potassium chloride buffer, pH 6.4. A separate aliquot of apo-Con A was used for each value of $[M_T]$. The data were taken several days after addition of Mn^{2+} ions. The curves through the data points show a fit of the model, Scheme III, to the experimental results, using the values K_{MPS} = 0.005 mM and K_{MMPS} = 0.02 mM.

concentration was 0.4 mM monomer (less than used in the dispersion runs, Figure 2a,b) chosen so that excess Mn^{2+} could be added without the relaxation rates exceeding the capabilities of the instrumentation. The pH was 6.4 to minimize the concentration of free Mn^{2+} ions required to fill the S1 sites. Mn^{2+} in different amounts was added to each sample, and the samples were left for several days at 25 °C, until all drifting had stopped. The relaxation rates for each sample were then measured at 0.02, 0.5, and 20 MHz at 25 °C. The results give a titration curve of relaxation rate as a function of total Mn^{2+} concentration; the long time for equilibrium to be reached after addition of Mn^{2+} requires that many samples be prepared simultaneously, rather than one sample altered by adding successive increments of Mn^{2+}. The titration data (Figure 5) are complex. It is seen that until about 1 equiv of Mn^{2+} per monomer is added, there is little free Mn^{2+} (by comparison of the data at 0.02 and 0.5 MHz). Addition of a second equivalent of Mn^{2+} still leaves very little free Mn^{2+}; the concentration of Mn^{2+} aquoion increases substantially only after more than 2 equiv is added. The second equivalent clearly binds to the protein to give two Mn^{2+} ions per monomer. Moreover, there must be an interaction between the Mn^{2+} ions at the two sites, since the relaxation rate at 20 MHz for two Mn^{2+} ions per monomer is less than for one.

Addition of Ca^{2+} to Mn^{2+}–Con A Equilibrium Complexes. Figure 6 shows the equilibrium relaxation rates of many of the samples shown in Figure 5 after addition of excess Ca^{2+} at 25 °C. The expectation is that Ca^{2+} will displace the second Mn^{2+} per monomer to form CMPE, leaving no free Mn^{2+} for any sample for which the total Mn^{2+} is less than the monomer concentration. For samples with higher concentrations of Mn^{2+}, the excess Mn^{2+} ions should be free in solution. The data should also be a check on the optical absorbance used to determine the protein concentration; two values in the literature differ by about 10% (Yariv et al., 1968) and Agrawal and Goldstein (1968) find $A_{280nm}^{1\%,1cm}$ of 12.4 and 11.4, respectively). The data, Figure 6, show a clear break, corresponding to a mean monomer concentration of 0.39 ± 0.02 mM, which agrees with an absorbance of 12.0 ± 0.4.

The relaxivities of the excess Mn^{2+}, derived from the samples with total Mn^{2+} concentrations greater than 0.4 mM, are 45, 23, and 10 $(mM\ s)^{-1}$ at 0.02, 0.5, and 20 MHz, respectively. These are to be compared with relaxivity values of 45,

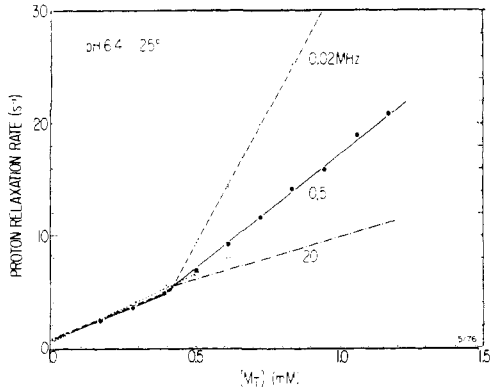

FIGURE 6: Solvent proton relaxation rate at three values of magnetic field, corresponding to Larmor frequencies of 0.02, 0.5, and 20 MHz, after addition of 1.67 mM Ca^{2+} ions to half the samples used for the data of Figure 5. The lines beyond values of $[M_T]$ greater than 0.4 mM are drawn with slopes corresponding to the relaxivities of Mn^{2+} aquoions derived from the data of Figure 1. The fact that the 20-MHz line falls below the data is compatible with about 2% of the excess Mn^{2+} binding at sites other than S1 and S2. The break in the data near 0.4 mM corresponds to an optical absorbance $A_{280mm}^{1\%,1cm} = 12.0 \pm 0.4$.

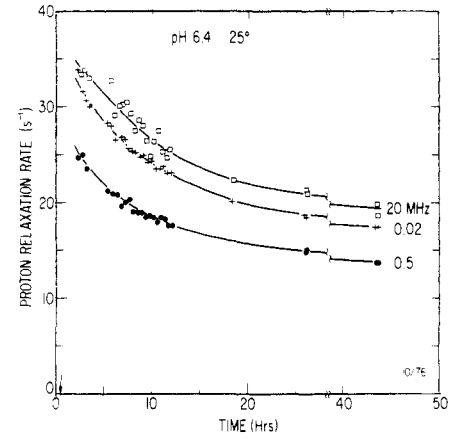

FIGURE 7: Time course of solvent proton relaxation rates at three values of magnetic field, corresponding to proton Larmor frequencies of 0.02, 0.5, and 20 MHz, after addition of 0.8 mM Mn^{2+} to 0.4 mM apo-Con A, pH 6.4. The sample corresponds to one used for the data of Figure 5.

23 and 8 $(mM\ s)^{-1}$ for free Mn^{2+} at these frequencies which were derived from the data of Figure 1. These latter relaxivity values are indicated by the slopes of the lines to the right of the break in the data in Figure 6. Both the relative and absolute relaxivities at 0.02 and 0.5 MHz indicate that the Mn^{2+} in excess of one ion per monomer is all free, within experimental error of a few percent. The higher value of 10 $(mM\ s)^{-1}$ for the 20-MHz data are compatible with approximately 2% of the excess Mn^{2+} bound at a third site with a high relaxivity (comparable to MPE, Figure 3a,b) and a large dissociation constant of about 10 mM. (We have since verified that Mn^{2+} binds weakly at a third site.) What is clear is that essentially all Mn^{2+} in excess of one per monomer is free in solution when S2 is occupied by Ca^{2+}.

Locking of Mn^{2+}-Con A Ternary Complexes. When sufficient Mn^{2+} is added to a sample of apo-Con A, the relaxation rates reach their equilibrium values only after a substantial time has elapsed. Representative data at 25 °C are shown in Figure 7 for a sample comparable to one of the many used in Figure 5. A point to note is the extremely long time required to reach equilibrium. The time course for locking of the Mn^{2+}-Con A ternary complex is longer at 25 °C than that of CMP at 5 °C for comparable metal content; changes in the relaxivity of the former are still observable after 24 h. Though we have not yet made a systematic study of the time dependence of this locking behavior, we find that if the amount of Mn^{2+} added is such that the occupancy of the second site is minimal, no significant drift of the relaxivity is observed, and little if any locking occurs.

Ancillary Experiments. We have performed a series of experiments designed to show that (1) the equilibrium ternary Mn^{2+}-Con A complex is in the same conformational state as CMPE; (2) on removal of the Mn^{2+} from the ternary complex at low temperatures the protein is left in this state, which is now metastable since there is no longer a metal at S2; and (3) this metastable state of apo-Con A will revert to its original conformation state after being held at 25 °C for a few days. The experiments were performed with several of the samples used for the titration data (Figure 5) (only half of these were used to obtain the data in Figure 6).

First, 0.8 mM Ca^{2+} was added at 5 °C to a sample with 1.2 mM total Mn^{2+}; for this sample essentially all protein molecules had two Mn^{2+} ions per monomer and were in their equilibrium state. The result was that, with no time delay (i.e., less than a few minutes), Ca^{2+} displaced 0.4 mM Mn^{2+} from the protein to give 0.8 mM free Mn^{2+} and 0.4 mM CMPE, as judged by the resulting relaxation dispersion. Thus, the equilibrium state of the protein with two Mn^{2+} ions per monomer is one which, though it is in rapid equilibrium with free Mn^{2+}, can bind Ca^{2+} rapidly and tightly, immediately forming CMPE. This is in marked contrast to the data of Figure 4a,b. This experiment was repeated on a sample with 0.4 mM total Mn^{2+}; for this sample essentially all protein molecules have one Mn^{2+} ion per monomer, and few have two. Upon addition of 1 mM Ca^{2+} to the sample at 5 °C, the relaxation rates behaved very much like the data in Figure 4b; there was an initial drop in the relaxation rate, followed by a slow decrease with time as Mn^{2+} was taken up to form CMPE. Thus, the equilibrium state of the protein with one Mn^{2+} per monomer is one which binds Mn^{2+} and Ca^{2+} weakly, and subsequently transforms slowly to the locked state.

Second, a sample with 0.4 mM protein and 1.2 mM total Mn^{2+}, one that had been allowed to equilibrate for several days at 25 °C so that essentially all protein molecules had two Mn^{2+} ions per monomer, was cooled to 5 °C, after which 1.3 mM EDTA was added. The EDTA, as expected, picked up all Mn^{2+}, and the dispersion became that of the Mn^{2+}-EDTA complex. Then 0.4 mM Mn^{2+} was added to the sample. These ions went immediately onto the protein, judged by the reappearance of a peak in the relaxation rate at 20 MHz, corresponding to a reasonably high relaxivity value. While still maintaining the sample cold, 1 mM Ca^{2+} was added. The protein immediately went to the low relaxing state CMPE; there was no time delay such as appears in Figure 4b, thus showing that the demetallized protein remains in the conformation state it was in when there were metals at S1 and S2.

Third, the above experiment was repeated with one alteration of the procedure. After EDTA was added, but before Mn^{2+} and Ca^{2+} were added, the sample was brought to 25 °C for 2 days and then cooled. In this case, the protein bound Ca^{2+} only weakly at first, and then the sample followed the usual time course toward the locked state (Figure 4b).

Monomer-Monomer Interactions. At the pH values and temperatures used here, the protein is expected to be dimeric. (At the highest pH value, it could be partially tetrameric.) There is the possibility, therefore, of cooperativity (negative or positive) in the binding of metals. We were sensitive to this possibility and always considered it whenever data were obtained for which the initial interpretation was not straight-

forward. The impression, admittedly qualitative, but derived from the experiments reported here as well as many others, is that we saw no evidence of such cooperative behavior. Binding of metal to sites on any monomer is independent of the state of occupancy of similar sites on the other monomer of a dimer (or tetramer).

Fragments. It is known that Con A dimers do not produce intact monomers upon dissociation (Wang et al., 1971). The proportion of fragments produced varies with the source, and differs for preparations of apo-Con A from Miles-Yeda and Sigma native Con A (Brewer, unpublished). Our results are independent of the source of the native material, and thus presumably insensitive to nicks in the monomeric units of the dimers.

Model and Data Analysis

All data in the previous section can be fit by a model for Con A which posits two conformation states for the protein monomer separated by a fairly high energy barrier (about 22 kcal M^{-1}), and which differ in the free energies of their ground states by only a small amount. Both the magnitude and sign of this difference depend on the occupancy of S2. We refer to the conformer which has the lower free energy when both S1 and S2 are occupied as the "locked" conformation, since metal binding is greater for this state; the other conformer we call "unlocked". For either conformer, S1 must be occupied before binding of metal at S2 can occur. Once S1 is occupied, Ca^{2+} or Mn^{2+} can bind at S2. Because of the high-energy barrier between the two conformations, the time to reach thermodynamic equilibrium can be very long.

Model for Binding of Mn^{2+}. The multiple equilibria between Mn^{2+} and protein are given by Scheme I. Here M refers

SCHEME I

$$\begin{array}{ccccc} M + P & \rightleftharpoons & MP + M & \rightleftharpoons & MMP \\ \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \\ M + PL & \rightleftharpoons & MPL + M & \rightleftharpoons & MMPL \end{array}$$

to free Mn^{2+} ions; P and PL to the apoprotein in the unlocked and locked conformations, respectively; MP and MPL to molecules of the two conformations with a single Mn^{2+} ion per monomer, bound at S1; and MMP and MMPL to molecules with two Mn^{2+} ions per monomer, bound at S1 and S2. The equilibria are rapid along the horizontal pathways, and slow along the vertical ones.

We define the following dissociation and equilibrium constants for Scheme I:

$$K_{MP} = [M][P]/[MP] \quad (1)$$

$$K_{MPL} = [M][PL][MPL] \quad (2)$$

$$K_{MMP} = [M][MP][MMP] \quad (3)$$

$$K_{MMPL} = [M][MPL]/[MMPL] \quad (4)$$

$$K_{LMMP} = [MMP]/[MMPL] \quad (5)$$

$$K_{LMP} = [MP]/[MPL] = K_{LMMP}(K_{MMP}/K_{MMPL}) \quad (6)$$

$$K_{LP} = [P]/[PL] = K_{LMMP}(K_{MP}/K_{MPL})(K_{MMP}/K_{MMPL}) \quad (7)$$

Model for Binding of Ca^{2+}. In the presence of both Mn^{2+} and Ca^{2+}, we have the additional equilibria shown in Scheme II and the corresponding dissociation constants:

SCHEME II

$$\begin{array}{ccc} MP + C & \rightleftharpoons & CMP \\ \downarrow\uparrow & & \downarrow\uparrow \\ MPL + C & \rightleftharpoons & CMPL \end{array}$$

$$K_{CMP} = [C][MP]/[CMP] \quad (8)$$

$$K_{CMPL} = [C][MPL]/[CMPL] \quad (9)$$

where C refers to free Ca^{2+}.

Mn^{2+}–Con A Equilibrium Complexes. Since the equilibrium ratios [P]/[PL], [MP]/[MPL], and [MMP]/[MMPL] are fixed by equilibrium constants, Scheme I can be simplified, at equilibrium, to Scheme III

SCHEME III

$$M + PS \rightleftharpoons MPS + M \rightleftharpoons MMPS$$

where

$$[PS] = [P] + [PL] \quad (10)$$

$$[MPS] = [MP] + [MPL] \quad (11)$$

$$[MMPS] = [MMP] + [MMPL] \quad (12)$$

The relaxivities of MPS and MMPS will then be weighted averages of the relaxivities of the respective locked and unlocked forms. We define two dissociation constants, in analogy with eq 1 and 3:

$$K_{MPS} = [M][PS]/[MPS] \quad (13)$$

$$K_{MMPS} = [M][MPS]/[MMPS] \quad (14)$$

It is straightforward to show that

$$K_{MPS} = K_{MP} \times \left[\frac{1 + (K_{MPL}K_{MMPL})/(K_{MP}K_{MMP}K_{LMMP})}{1 + K_{MMPL}/(K_{MMP}K_{LMMP})}\right] \quad (15)$$

$$K_{MMPS} = K_{MMPL}\left[\frac{1 + (K_{MMP}K_{LMMP})/K_{MMPL}}{1 + K_{LMMP}}\right] \quad (16)$$

One can derive values for K_{MPS} and K_{MMPS} from the titration data (Figure 5) once the relaxivities at the three fields are known for both the MPS and MMPS complexes, since the observed relaxation rates can then be used to calculate the concentrations of the several species. Approximate values for the relaxivity of MPS were taken from the data of Figure 2b, which are for the sample with the greatest excess of protein. This value will be exact if either MP and MPL have the same relaxivities, or if [MP] $\gg$ [MPL] at equilibrium. Approximate values for the MMPS relaxivities can be deduced from the high Mn^{2+} concentration end of the titration data. The solid lines (Figure 5) are a best fit to the titration data, and were obtained by successively refining trial values for relaxivities and dissociation constants until an optimal fit was obtained. The final relaxivity values were 50, 50, and 100 $(mM\ s)^{-1}$ for MPS and 24, 24, 31 $(mM\ s)^{-1}$ for MMPS at 0.02, 0.5, and 20 MHz, respectively. (Note that the relaxivity values for MMPS are for a monomer containing two Mn^{2+} ions.) The values found for the dissociation constants at pH 6.4, 25 °C, are K_{MPS} = 0.005 mM; K_{MMPS} = 0.02 mM. From the fit, one can also compute the concentrations of [M], [MPS], and [MMPS] as functions of total Mn^{2+} added. These results are illustrated in Figure 8.

The Mn^{2+} titration data clearly show that Mn^{2+} binds at a site in addition to S1, and that the results can be modeled assuming that this second site is formed only after S1 is occupied. Moreover, binding of the second Mn^{2+} influences the relaxivity of the first, since the two together have a lower relaxivity than a single Mn^{2+} at S1. Data indicating that the second binding site is S2 are interpreted below.

Ca^{2+}–Mn^{2+}–Con A Equilibrium Complexes. The dispersion curves for CMPE (Figure 2a,b), the titration data (Figure

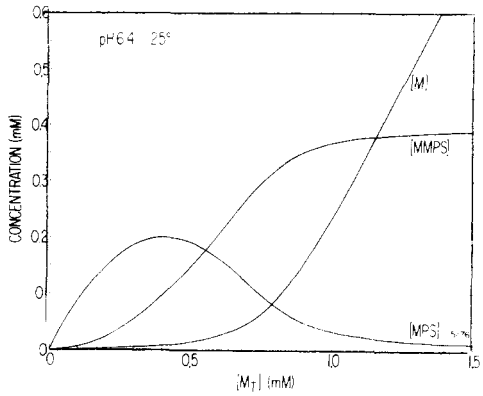

FIGURE 8: Values obtained for the dependence of the equilibrium concentrations of the Mn^{2+} aquoion [M], the binary Mn^{2+}-Con A complex [MPS], and the ternary Mn^{2+}-Mn^{2+}-Con A complex [MMPS] as a function of total Mn^{2+} concentration [M_T], by applying the model, Scheme III, to the data of Figure 5. The values K_{MPS} = 0.005 mM and K_{MMPS} = 0.02 mM were used.

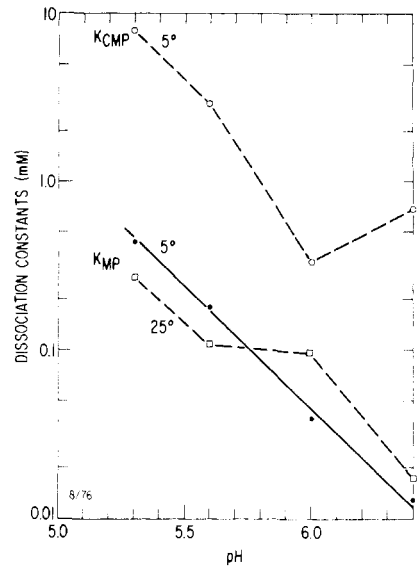

FIGURE 9: Values for the dissociation constants K_{MP} and K_{CMP} as a function of pH. Values for 5 °C are derived from data as in Figures 4a,b and 2a,b for 25 °C.

6), and data not illustrated show that, for all values of pH considered, all free Mn^{2+} is taken up when there is excess protein and Ca^{2+}. The data of Figures 5 and 6 show unequivocally that, when Ca^{2+} is bound at S2, the second Mn^{2+} per monomer is no longer bound. We take this fact, together with the interaction of the two Mn^{2+} ions per monomer indicated by the changes in relaxivities, as a strong argument that Ca^{2+} displaces Mn^{2+} from S2. It is possible that the second Mn^{2+} binds elsewhere, at a site which has an allosteric interaction with Ca^{2+} at S2 and Mn^{2+} at S1. However, other known metal-binding sites are remote from S1 and S2 (Becker et al., 1975; Hardman and Goldstein, 1977) and binding of various metals at these sites does not influence the relaxivity of Mn^{2+} bound at S1 (Sherry and Cottam, 1973).

Locking of Ca^{2+}-Mn^{2+}-Con A Complexes. The data (Figures 4a,b) indicate the time course of the relaxation rates at different values of magnetic field as the samples approach thermal equilibrium from the nonequilibrium states in which they were prepared. The explication in terms of our model is straightforward. In the absence of Ca^{2+}, and with only a small amount of total Mn^{2+} added so that all bound Mn^{2+} may be considered to be at S1, the protein is in the unlocked conformation; i.e., as MP rather than MPL. Addition of Ca^{2+} causes immediate formation of some CMP (a low relaxivity species), with its concentration determined by the rather large dissociation constant K_{CMP}, eq 8. These CMP molecules are not at equilibrium; their more stable conformation is the locked one, and we find that they decay to their ground state according to

$$d[CMPL]/dt = [CMP]/T_L \quad (17)$$

That is, CMP molecules decay according to first-order kinetics with a rate constant $1/T_L$ and the equilibrium is so much in the direction of CMPL that the back rate may be ignored.

Once the differential equation controlling the time dependence is known, and the initial conditions are defined, T_L and the time dependencies of the six unknown quantities [M], [C], [P], [MP], [CMP], and [CMPL] can be determined using eq 1, 8, 17, and eq 18–21 below.

$$[M_T] = [M] + [MP] + [CMP] + [CMPL] \quad (18)$$

$$[C_T] = [C] + [CMP] + [CMPL] \quad (19)$$

$$[P_T] = [P] + [MP] + [CMP] + [CMPL] \quad (20)$$

$$1/T_1 = R_P[P_T] + R_M[M] + R_{MP}[MP] + R_{CMP}[CMP] + R_{CMPL}[CMPL] + 1/T_{1w} \quad (21)$$

Here $[M_T]$, $[C_T]$, and $[P_T]$ are the total concentrations of Mn^{2+}, Ca^{2+}, and protein, respectively, and R with various subscripts indicates the relaxivity values of the respective components of the solution at a given value of magnetic field. $1/T_{1w}$ is the contribution of the buffer to $1/T_1$. The initial condition taken is that [CMPL] = 0 (or else very small, with a value to be determined by trial for a best fit to the data). Then eq 1, 8, and 18–20 expressing the relatively rapid equilibria among the five remaining concentrations are solved, a value for [CMP] is obtained, and, using a rough estimate for T_L and a small time step, a value is computed for the incremental change in [CMPL] using the differential equation, eq 17. The remaining five equations are solved again for new values of the five concentration variables, and the process is iterated until the values of the variables stop changing with time. At each iteration, $1/T_1$ is computed for each value of field using eq 21 and the known relaxivities of the various components of the solution. The process is repeated for different values of T_L until a best fit of the computed values of $1/T_1$ to the data is obtained.

To perform the fitting procedure, values for the parameters K_{MP} and K_{CMP} are required at each pH. K_{MP} values are obtained from data as in Figure 4a,b, just before Ca^{2+} is added, using the definition, eq 1, and the facts that $[M_T]$ and $[P_T]$ are known and that [M] can be obtained from the difference in relaxivities at 0.02 and 0.5 MHz. Similarly, K_{CMP} can be obtained from the initial drop in the relaxation rates upon Ca^{2+} addition (which requires extrapolation of the data back to zero time). The values so obtained for K_{MP} and K_{CMP} (at 5 °C) are shown in Figure 9. In the fit, we assume (as mentioned above) that at equilibrium [P] $\gg$ [PL] and [MP] $\gg$ [MPL], so that K_{MP} can be used for K_{MPS}. This has been partially justified above, and will be justified in detail below. The results are shown by the solid and dashed lines in Figure 4a,b. The time dependencies of the concentrations of relevant species are also shown, as are the computed variations of the relaxation rates at the three fields. The relaxivity values at 5 °C were obtained from data as in Figure 3a,b: for R_{MP} we used the MPE results for the Ca^{2+}-free samples, and for $R_{CMP} = R_{CMPL}$ (see below) we used the CMPE data. R_M values were obtained from Figure 1, R_P from Figure 2a,b and $1/T_{1w}$ was measured directly. The results are T_L = 0.285 ± 0.015 h and is independent of pH, within the stated uncertainty.

The foregoing procedure for fitting the model to the data is not unique. We could have first obtained values for the concentration of free Mn^{2+} as a function of the time from the differences in the relaxation rates at 0.02 and 0.5 MHz (Figures 4a,b). Then the five equations (1, 8, 18–20) could have been solved, at each value of time, for the five unknowns [C], [P], [MP], [CMP], and [CMPL], and the results compared with eq 17 both to test its validity and to obtain a value for T_L. Thus, any experimental technique that can measure the concentration of free Mn^{2+} fairly rapidly can be used to study the locking of CMP.

Several minor points must be noted. The fact that R_{CMP} and R_{CMPE} have the same value (or very closely so) is evident from the experiment in which sufficient total Ca^{2+} was added to force the equilibrium MP + C $\rightleftharpoons$ CMP totally to the right. In this instance, the relaxation rates at all fields dropped immediately to their final values and remained independent of time. Relaxation measurements under these conditions cannot directly detect the conversion of CMP to CMPL; only the fact that EDTA removed metals at short times and not at long times proves that locking was going on. Another point that indicates that CMP and CMPL have essentially the same relaxivity is the fact that the time-dependent data can be fit at all fields by a single value of T_L.

At the two lower pH values, fitting of the data indicated the formation of a small amount of CMPL immediately upon addition of Ca^{2+} (cf. Figure 4a). This could indicate the presence, at equilibrium and before addition of Ca^{2+}, of a small amount of PL, MPL, or MMPL (or adventitious Ca^{2+}), since free CMPL would form rapidly from these species. We have incorporated the observed initial values of [CMPL] in the fits, but have not included the small effect this would have when computing the dissociation constants (Figure 9). Nor has the small amount of binding of Mn^{2+} at S2 under these conditions been taken into account.

The relatively small values of [CMPL] found immediately upon Ca^{2+} addition at the lower pH values, and the absence of CMPL at the higher pH values, justify the assumptions made in reducing these data that [P] $\gg$ [PL] and [MP] $\gg$ [MPL] at equilibrium. Otherwise Ca^{2+} would have gone immediately onto any MPL that existed at equilibrium to form CMPL with no time delay. This assumption is also supported by data (not illustrated) where locking is observed for two samples identical in all respects except for the amount of added Ca^{2+}. Not only are the time courses of the relaxation rates fit by the same values of T_L, but the computed values of K_{CMP} are the same for both samples. Neither would not be true if significant amounts of Ca^{2+} were immediately taken up to form CMPL. This result is also relevant to the fit of the titration curve (Figure 5) to Scheme III. It allows the value for K_{MPS} (eq 13) to be equated with K_{MP}. However, since K_{LMMP} is as yet unknown, an analogous association cannot be made for K_{MMP}.

One remark is in order concerning the model. In Schemes I and II, we have assumed sequential occupation by metals at sites S1 and S2 for both conformations of the protein. The requirement is well established for the unlocked conformation, i.e., the upper set of equilibria, Schemes I and II. However, the data to date do not require this restriction for the locked conformation, i.e., the lower set of equilibria, but the assumption does simplify the application of the model to the interpretation of the data. If necessary, the model can readily be generalized without changing its qualitative aspects.

Discussion

Using measurements of proton relaxation dispersion to study the interaction of Mn^{2+} and Ca^{2+} ions with Con A, we have demonstrated the existence of two conformation states of the protein (which we refer to as "unlocked" and "locked"). Apo-Con A prepared by acid demetallization of native Con A is essentially all in the unlocked conformation, symbolized by P in Scheme I. Limited amounts of Mn^{2+} added to a solution of P bind at S1 to form MP. This binary complex is characterized by a relatively large and pH-dependent dissociation constant, K_{MP} (see below); rapid exchange of Mn^{2+} ions; and a pH-independent relaxivity which is relatively high at all fields, with a pronounced peak near 15 MHz (Figure 3a,b). With S1 occupied, Ca^{2+} ions can bind at S2 to form the ternary complex in the unlocked conformation, CMP (Scheme II). The dissociation constant of Ca^{2+} for this complex, K_{CMP}, is also large (about ten times K_{MP}) and pH dependent, and Ca^{2+} is in rapid exchange with MP. Again, the relaxivity is pH independent, but relatively low, a factor 5 to 8 less than that of MP, depending on the value of magnetic field, with a slight peak near 15 MHz (Figure 3a,b).

The initial conformation of CMP, however, is not an equilibrium conformation and, with time, the CMP complex undergoes a first order transition to the locked conformation, CMPL (Figure 4a,b). The value of the rate constant for the transition is a function of temperature, but is independent of pH. On the other hand, the overall time for the resulting variations in relaxation rates depends on the concentrations of Ca^{2+}, Mn^{2+}, and protein, and on pH as well, because of the pH dependence of K_{MP} and K_{CMP}. The locked ternary complex, CMPL, has essentially the same dispersion as CMP, but binds Ca^{2+} and Mn^{2+} very strongly; when dialyzed against large volumes of buffer at room temperature for several days, CMPL slowly loses its metals and ultimately returns to the unlocked apoprotein P. It should be noted that investigations of the saccharide binding and agglutination activity of Con A are normally done with the CMPL form of the protein.

We have found that S2, previously thought to bind only Ca^{2+} and Cd^{2+}, also binds Mn^{2+} ions. At sufficiently high concentrations, Mn^{2+} forms a ternary complex with Con A in the unlocked conformation, MMP, with Mn^{2+} at S1 and S2 (Scheme I). The dissociation constant for Mn^{2+} at S2, K_{MMP}, is large and comparable to K_{CMP}; the exchange of Mn^{2+} with MMP is rapid; and the relaxation rate per bound Mn^{2+} ion is intermediate between MP and CMP (see below). MMP is also a nonequilibrium conformation, and undergoes a change to the locked conformation, MMPL (Figure 7), with a locking rate far smaller than for the CMP $\rightarrow$ CMPL transition. The relaxivity of MMPL is substantially lower than that of MMP and, in contrast with Ca^{2+} in CMPL, the off-rate of Mn^{2+} from S2 is high. It should be remarked here that the two Mn^{2+} ions per monomer in MMP and MMPL may be magnetically coupled because of their proximity; i.e., the relaxivity contribution of Mn^{2+} at S1 may be different depending upon whether Ca^{2+} or Mn^{2+} is at S2. We have no information on this point as yet.

The fact that Mn^{2+} is in rapid exchange with MMPL has allowed us to prepare PL, the apoprotein in the locked conformation, simply by addition of EDTA to MMPL at 5 °C. PL is a long-lived, nonequilibrium species, and we have shown that it can be maintained for days at 5 °C, as evidenced by the reintroduction of Ca^{2+} and Mn^{2+} to form CMPL along the pathway PL $\rightarrow$ MPL $\rightarrow$ CMPL with no time delay (Schemes I, II). A sample of PL kept at room temperature for about 24 h reverts to P, as evidenced by the fact that upon addition of Mn^{2+} and Ca^{2+} the sample is observed to follow the pathway P $\rightarrow$ MP $\rightarrow$ CMP $\rightarrow$ CMPL, with the expected slow time course for the last step. Thus, we have a model for binding of

TABLE I: Dissociation Constants of Several Metal Ion-Con A Complexes, Obtained from the Present Work and from the Literature.[a]

pH	K_{MP} (mM) 5 °C	K_{MP} (mM) 25 °C	K_{CMP} (mM) 5 °C	K_{MMPS} (mM) 25 °C	K_{MMP} (mM) 25 °C
5.3	0.43[b]	0.27[b]	8.0[f]	~0.8[h]	
	~0.5[d]	0.36[c]	~0.3[g]		
5.6	0.18[b]	0.11[b]	2.9[f]	~0.5[h]	
		0.14[c]			
		0.050[e]			
		0.19[e]			
6.0	0.04[b]	~0.1[b]	0.33[b]		
		0.10[c]			
6.4	0.01[b]	~0.02[b]	0.70[f]	~0.07[h]	~0.5[j]
		~0.03[c]		0.02[i]	
		0.005[i]			

[a] Values indicated as approximate may vary a factor of four over the extremes of their range of uncertainty; the uncertainties of values of other constants derived from the present work is typically one or two in the last significant digit. [b] From the data, Figure 2a-b, uncorrected for a small amount of binding of Mn^{2+} at S2. [c] Corrected for binding of Mn^{2+} at S2, using the results in Figure 3a-b, and analogous data at other pH values, (see text). [d] Shoham et al. (1973), at 4 °C and pH 5.2. [e] Sherry and Cottam (1973); see text. [f] From the data, Figures 4a-b, and analogous data at other pH values. [g] Kalb and Levitzki (1968), for Ni^{2+} at S1, at 3 °C and pH 5.2 (see text). [h] Estimated, using the results in Figures 3a, and analogous data at other pH values. [i] From the data, Figure 5. This value is a good estimate for K_{MMPL}. [j] From the data, Figure 7.

Mn^{2+} and Ca^{2+} to Con A which involves three different metal-protein complexes for each of the two protein conformations, for a total of six metal-protein complexes and two states of the apoprotein. Only half of these states are equilibrium ground states, but we are able to prepare all of them, several of which are very long-lived under proper conditions. A detailed analysis of the foregoing observations is given below.

Metal Dissociation Constants. There are six independent dissociation constants that characterize the binding of Mn^{2+} and Ca^{2+} to the two conformations of Con A: K_{MP} and K_{MPL}, the dissociation constants for Mn^{2+} bound at S1 to the unlocked and locked conformers respectively; K_{MMP} and K_{MMPL}, for Mn^{2+} bound at S2; and K_{CMP} and K_{CMPL}, for Ca^{2+} bound at S2. (There is also one additional independent equilibrium constant that gives the ratio of the concentrations of the two conformers at equilibrium.) We have obtained values for K_{MP} and K_{CMP} at 5 °C and K_{MP} at 25 °C, over the pH range 5.3-6.4, from data as in Figures 2a,b and 4a,b; the results are presented in Figure 9 and Table I. These values are uncorrected for the small amount of Mn^{2+} at S2; including an approximate correction gives the results at 25 °C for K_{MP} and K_{MMP} in Table I. The computations indicate that about 12% of the protein with bound metals is MMPL for those samples at pH values other than 6.0. We have also included in Table I an estimate for K_{MMPL} at pH 6.4 and 25 °C obtained from the value of K_{MMPS} (Figure 5) and a rough estimate for K_{MMP} from the data of Figure 7. The binding of Ca^{2+} in CMPL is so strong in the pH range studied that we cannot measure K_{CMPL}. Finally, we have no estimate of K_{MPL}; we do not know whether it is greater or less than K_{MP}. However, since we can prepare apoprotein in the locked conformation, it should be possible to obtain values for K_{MPL} in the future.

Other authors have reported values for some of the dissociation constants, and these results are also in Table I. Shoham et al. (1973) list a value for K_{MP} which, within their error, agrees with our results. However, Kalb and Levitzki (1968) had difficulty obtaining a well-defined value for the dissociation constant of Ca^{2+} at 4 °C from, in their case, the Ni^{2+}-containing protein. They added Ca^{2+} in increasing amounts, using equilibrium dialysis for a *fixed time* to measure Ca^{2+} binding. From curvature in the resulting Scatchard plot, they suggested that there was heterogeneity in the affinity constant. By contrast, we infer from their results that more of the Ca^{2+}-Ni^{2+}-Con A complex was formed with increasing Ca^{2+} concentration, thereby allowing an increasing amount of the locked complex to form in successive experiments. Their use of the same elapsed time for each dialysis experiment obscured the underlying time-dependent phenomena.

Sherry and Cottam (1973) derived a value for K_{MP} of 0.050 mM at 24 °C, pH 5.6, using a combination of proton relaxation and electron-spin resonance measurements. (Their "monomer" corresponds to our dimer, but that does not alter the value of the dissociation constant.) However, from the known relaxivities of free and bound Mn^{2+}, we derive the value K_{MP} = 0.19 mM from the titration data of Sherry and Cottam, their Figure 2.

The pH dependence of the binding of Mn^{2+} to apoprotein in the pH range 5.3-6.4 suggests competition with a proton for the one histidine residue ligand at S1. There is an equally large pH dependence of the binding of Ca^{2+} to S2 for the unlocked conformation of Con A. The main pK for Ca^{2+} dissociation from CMPL is near 4.5, and, judging from the titration of Con A activity, it appears to involve one proton (cf. Sherry et al., 1975; Figure 2). Since the S2 site of Con A contains only carboxylic acid ligands, the contributing pK for binding of Ca^{2+} at S2 would appear to be associated with one of the carboxyl groups. Whatever the ionizations are that are responsible for the pH dependence of metal binding, once the metals are bound there is no observable dependence on pH of the relaxivities of the Mn^{2+}-protein complexes MP, CMP, and CMPL, nor of the rate constant for the change of conformation of the protein from the unlocked to the locked conformation.

Kinetic Effects upon Addition of Ca^{2+} to Mn^{2+}-Con A. The data, as in Figure 4a,b, show that the time course of locking of a sample of MP to which Ca^{2+} is added depends upon pH, although the derived transition rate of a CMP molecule to CMPL is pH independent. The physical basis underlying this observation is that the binding of Ca^{2+} and Mn^{2+} is greater at higher pH values; thus, a greater fraction of the unlocked conformers have metals initially, and therefore the net *velocity* of the reaction leading to the CMPL equilibrium state is greater. (A similar pH dependence should be observed in locking of MMP, though we have not investigated this in detail; there may, of course, be an additional pH dependence of the locking behavior of MMP were the rate constant pH dependent in this case.)

For the pH 6.0 and 6.4 samples, the binding of Mn^{2+} to P is sufficiently strong so that there is little free Mn^{2+}. The initial rate of decay of the relaxation rate, in these samples, is then equal to the product of the locking rate of CMP, the initial concentration of CMP, and the difference in the relaxivities of MP and CMPL. For these samples, the [CMP] is about [MP]/3, as estimated from K_{CMP}, and the initial time constant for decay is therefore somewhat under 1 h (i.e., $3T_L$) at 5 °C. The time course of the decay is roughly exponential until a significant amount of CMPL builds up and MP becomes depleted. For the pH 5.3 sample (Figure 4a) the initial value of [CMP], 0.03 mM, is a small fraction of the initial value of [MP], 0.13 mM, which in turn is less than half the total Mn^{2+} concentration. The intrinsic initial decay rate (Figure 4a) is

then of order $0.1/T_L$. At higher pH values, the change of relaxation rate is due solely to conversion of MP to CMPL, i.e., to a shift from a high to a low relaxivity form of bound Mn^{2+}. At lower pH values, there is also a time-dependent change in the free Mn^{2+} concentration, and this contributes to the variation of the observed relaxation rates with time.

It is possible to extract an accurate value for T_L at 23 °C by applying our model to the results of Grimaldi and Sykes (1975). These authors measured the nuclear magnetic resonance absorption intensity of water protons at 40.5 MHz in a stopped-flow chamber, as a function of time after mixing a sample of apo-Con A with buffer containing Mn^{2+} and Ca^{2+} ions. The bulk of their data that are comparable to ours were obtained from samples at pH 5.3 and 23 ± 1 °C (Grimaldi et al., 1972) which contained 0.14 mM protein monomer, 0.5 mM total Mn^{2+}, and a variable concentration of Ca^{2+} ranging from 1 to 25 mM. For these conditions, it is a reasonable approximation to consider that all protein molecules have Mn^{2+} ions at S1; therefore, when Ca^{2+} is added, CMP will convert to CMPL with an initial velocity proportional to the initial value of [CMP] which depends only on K_{CMP} and the concentration of free Ca^{2+}. The magnetic resonance absorption intensity is proportional to T_2, the spin–spin or transverse relaxation time of solvent protons (Grimaldi et al., 1972). It is adequate to consider T_2 to be related to T_1 in a simple way: T_2 of bound Mn^{2+} at 40 MHz has approximately the same value as T_1 at low fields (Koenig et al., 1971). The time-dependent absorption intensity observed by Grimaldi and Sykes, which they treat as a purely phenomenological parameter, is then analogous to our high pH case, since all S1 sites are occupied because of the large excess of Mn^{2+}. There is an initial uptake of Ca^{2+} to form CMP, which then converts to CMPL; more Ca^{2+} is then taken up by MP, resulting in a continuous conversion of molecules of high relaxivity to ones of low relaxivity. The absorption then should increase with time, as it does. There will be, in addition, a large background contribution due to free Mn^{2+} ions which will be essentially constant in time. For the experimental conditions chosen, one expects (from our model) an initial rate for the change of absorption intensity that is proportional to the initial value of [CMP], and thus a plot for this rate as a function of total Ca^{2+} concentration should reproduce the binding curve of Ca^{2+} to [MP]. This is indeed what Grimaldi and Sykes observe (their Figure 2), from which data we derive $T_L = 95$ s (using the saturation value of their initial velocity) and a value for K_{CMP} of about 1 mM at pH 5.3 and 23 °C. This value of T_L at 23 °C, together with our value of 0.29 h at 5 °C, allows us to compute an activation energy of 21.7 kcal M^{-1} for the height of the energy barrier that separates CMP and CMPL. Using the usual Eyring-type model for rate processes, we compute a frequency prefactor of 2×10^{14} Hz. Additionally, combining the forward time constant of 95 s with a time of about 1 day to remove Ca^{2+} at 25 °C by dialysis gives an energy difference between the two conformers, CMP and CMPL, of about 3.7 kcal M^{-1} or an energy difference of about one hydrogen bond.

There are other details of the results of Grimaldi and Sykes that we can interpret. They plot the magnitude of the change of absorption from zero to "infinite" time, as well as the absorption intensity at infinite time, as a function of total Ca^{2+} added (which is always in excess of the monomer concentration). We would expect this from our model, since all protein ultimately becomes CMPL. The absorption then depends only on this final [CMPL] value plus a contribution from free Mn^{2+}. The magnitude of the absorption change is, in terms of our model, the magnitude of the relaxation rate that remains to change as locking progresses after the initial drop upon adding Ca^{2+} (cf. our Figure 4a). It is well under 5% of the total intensity, in contrast to our data (Figure 4a,b) and of course, depends on Ca^{2+} concentration. We add less Ca^{2+} than do Grimaldi and Sykes, and therefore observe a larger effect. The more Ca^{2+} added, the more CMP formed, and the closer will be the initial relaxation rate to the final one, since CMP and CMPL have essentially the same relaxivities. Therefore, the magnitude of the change they observe should become smaller, as is the case.

Grimaldi and Sykes concluded from their data that Ca^{2+} and Mn^{2+} are initially in rapid exchange with a conformation of the protein that subsequently undergoes a change after Ca^{2+} binds, with which we agree. However, they hypothesize (incorrectly, in our view) that Ca^{2+} *catalyzes* this change, which they try to prove by incubation experiments. A solution of apoprotein is incubated with 3 equiv of Mn^{2+} and 0.3 equiv of Ca^{2+}; after variable times, ranging to several days, more than a 100-fold excess of Ca^{2+} is added. The results are complex, but the important result is that, after long incubation, there is an initial decrease in the absorption intensity upon Ca^{2+} addition, and then the usual increase of the initial absorption rate equal to their previous rates at high Ca^{2+} concentration. They offer no explanation, but in terms of our model the phenomena results from binding of Mn^{2+} at S2. After 4 days, Mn^{2+} at S2 has itself produced some MMPL (cf. their Figure 11). Subsequent addition of excess Ca^{2+} promptly displaces the Mn^{2+} from the S2 sites of MMPL molecules, producing an immediate increase in relaxation rate due to the initial increase of free Mn^{2+} and making it *appear* that a small amount of Ca^{2+} has acted as a catalyst of the conformation change; recall that Ca^{2+} can go directly onto the locked form with no time delay, and that T_2 of free Mn^{2+} is short. Other Ca^{2+} meanwhile binds to (unlocked) MP to form CMP which then locks at the usual rate.

Barber and Carver (1973) have reported a time dependence of T_1 of water protons after addition of Mn^{2+} to solutions of untreated commercial Con A at 24 °C. Their tests indicated that about 5% of the monomers contained Mn^{2+} ions, and it is not known whether other metal ions (e.g., Zn^{2+}) were at the S1 sites of the other protein molecules, or whether they were demetallized. The great heterogeneity of commercially available Con A has been demonstrated by Uchida and Matsumoto (1972). We have no explanation for the time-dependent data of Barber and Carver, unless they observed an exchange of Mn^{2+} with another metal ion on the protein for which the rate-limiting step is the off-rate. (In a subsequent paper, Barber and Carver (1974) imply that they could not reproduce their earlier time-dependent results, and suggest that perhaps adventitious Ca^{2+} could have caused the anomolous observations.) Their 1973 data are consistent with ours only after they add Ca^{2+}; the relaxivities at their one frequency (32 MHz) and one temperature are in agreement with ours.

Kinetic Effects Upon Addition of Mn^{2+}. The data (Figure 7) show clearly that locking occurs when Mn^{2+} binds at both S1 and S2. However, a quantitative description of the kinetics of the underlying conformation change in terms of our model is more complex than for the analogous CMP → CMPL transition. The major complication is that the relaxivity dispersion of MMP is not the same as that of MMPL (in contrast to CMP and CMPL), a fact that can be readily deduced from the data (Figure 7). From the difference in relaxation rates at 0.02 and 0.5 MHz shortly after addition of Mn^{2+}, one calculates a free Mn^{2+} concentration of about 0.3 mM. This means that 0.5 mM of bound Mn^{2+} has an average relaxivity, per Mn^{2+} ion, of about 45 and 70 $(mM\ s)^{-1}$ at 0.02 and 20 MHz, respectively. Assuming that all protein has at least one Mn^{2+}

bound, corresponding to the minimum amount of MMP, the relaxivity calculated for MMP is about 60 $(mM\ s)^{-1}$ at 0.02 and somewhat less at 20 MHz, far higher than the corresponding values of 24 and 31 for MMPE. (Assuming all the bound Mn^{2+} is in MMP molecules makes the relaxivities still higher.) It will require additional data to find accurate relaxivity values for MMP and, from these, to derive values for K_{MMP} and K_{LMMP} and, finally, to obtain a precise description of the locking of MMP. Qualitatively, T_L for MMP is at least an order of magnitude longer than T_L for CMP at 25 °C.

Ancillary Experiments and the Model. The ancillary experiments were chosen to test some predications of the model (Schemes I and II). In the first experiment, Con A was prepared in the MMPL state at 25 °C. Addition of Ca^{2+} at 5 °C displaced all Mn^{2+} from S2 to form CMPL immediately, with no subsequent drift in relaxation rates. This indicates that the on-rate of Ca^{2+} is high. Its very low off-rate from CMPL, determined from dialysis experiments, means that the binding of Ca^{2+} to CMPL is extremely tight and that at equilibrium [CMPL] $\gg$ [CMP]. The lack of drift after Ca^{2+} addition shows that there was essentially no MP present initially.

By contrast, in the second experiment with less Mn^{2+} added, mostly MP was generated. Addition of Ca^{2+} made the equilibrium proceed via the pathway MP → CMP → CMPL, the last step taking a substantial time.

The third experiment was a repeat of the first to produce MMPL, after which all metal was removed by addition of EDTA at 5 °C, leaving PL. The equilibrium configuration for this sample would be for PL to have reverted to P; however, our hypothesis, which was substantiated, was that the time to reach equilibrium would be very long at 5 °C. That PL and not P was produced is indicated by the fact that readdition of a small amount of Mn^{2+} (such that occupancy of S2 was negligible) immediately formed a high relaxivity species, and that subsequent addition of Ca^{2+} formed CMPL with no time delay. This also shows that MPL is a high relaxivity species.

The fourth experiment was a repetition of the third, except that the sample was kept at 25 °C for about 2 days after EDTA addition in order to allow PL to convert to the (equilibrium configuration) P. With subsequent addition of metals at 5 °C, the sample followed the pathway P → MP → CMP → CMPL with the expected time delay for the last step.

The ancillary experiments were done with sufficient precision to establish the fact that the various forms of the protein (i.e., apo- and metal-containing) can be produced in either of two conformations by judicious cycling of the various reactions. The precision is not yet adequate to obtain values for the relaxation dispersions of all species, nor values for all the six independent equilibrium constants, eq 1–4, 8, 9. However, since we can produce both conformations of apo-Con A, we can study the binding of metals to both forms, as well as their biological activity, which could depend more on conformation than metal content.

Relation to X-Ray Data. The pH independence of the relaxivities of CMP and CMPL, and of the rate of transition from CMP to CMPL, contrasts with the significant pH dependence of the binding of metals to the unlocked conformers. This suggests that the fundamental conformation change that distinguishes the unlocked and locked conformers occurs somewhat remote from S1 and S2. Jack et al. (1971), from x-ray studies, found that crystals of demetallized protein and native protein have different space groups, that neither the separate addition of Mn^{2+} nor Ca^{2+} ions to apo-Con A crystals alters the space group, but that the addition of both ions results in cracking of the crystals and alteration of the structure to one indistinguishable from the native (i.e., the Mn^{2+}- and Ca^{2+}-containing) protein. Using rotational functions to compare the electron densities of the two space groups, they concluded that the "structures of native and demetallized concanavalin A are closely related although not isomorphous". Becker et al. (1976) and, more recently, Shoham et al. (Yonath, private communication) have concluded more complete x-ray analyses of single crystals of apo-Con A. The observed differences in the structures of demetallized and native proteins are mainly in the metal-binding region. Shoham et al. also report side-chain motions, different for each monomer, which compensate for the changes in charge at S1 and S2 when Mn^{2+} and Ca^{2+} are removed.

We associate the unlocked conformation of Con A with the space group of the apoprotein crystals, and the locked conformation with the space group of the native crystals. Addition of Mn^{2+} to the S1 sites of crystalline apo-Con A causes changes in position of some side chain and backbone groups, but does not initiate the fundamental change that distinguishes the locked conformation from the unlocked. The nature of this change is unknown. However, Hardman (1973) and Reeke et al. (1975) have indicated an apparent cis amide bond between residues Ala-207 and Asp-208, which are near S2 and the saccharide-binding site, but fairly distant from S1. Cis amide bonds are rare in proteins, and, indeed, all known instances involve an X-proline bond (Wüthrich, private communication; Wüthrich and Grathwohl, 1974). Residue 206 in Con A is proline, and whether the displacement of the cis bond assignment to His-205–Pro-206 is consistent with the present x-ray data will have to wait upon further refinement of these data, according to Hardman (private communication). Evidence that a cis–trans isomerization of a proline amide bond may be involved in the transition between the locked and unlocked forms of Con A comes from consideration of the value of the activation energy of 21.7 kcal M^{-1} that we have measured for this transition. This value agrees very well with the activation energies found for cis–trans isomerization of proline-containing dipeptides of 19.8 kcal M^{-1} found by Brandts et al. (1975) and 22.3 kcal M^{-1} found by Roques et al. (1977), and 20.6 kcal per mol of proline found for polyproline by Steinberg et al. (1960). Moreover, we have shown that protein can be prepared in either conformation, with and without metals, and that the metastable states can be quite long-lived. The lifetimes are comparable to those observed by Brandts et al. (1975) for dipeptides containing proline amide bonds. In addition, Thomas and Williams (1972) and Zimmerman and Scheraga (1976) have shown that the difference in the ground-state energies of the cis and trans conformations of dipeptides is small, as we find, but depends upon the combined contributions of several larger interactions, of different signs, that are sensitive to the distribution of electrostatic charges.

All the above leads us to attribute the difference between the locked and unlocked conformations of Con A to a cis–trans isomerization of a proline amide bond in Con A, possibly Pro-206, and thus to a difference in secondary rather than tertiary structure of the protein. This transition would satisfy the criteria that the data impose: the high-barrier and low-energy difference, the need for a change more specific than the general motions observed when Mn^{2+} above is added to apoprotein crystals, (e.g., an alteration of the secondary rather than the tertiary structure of the monomer), and the fact that the CMP → CMPL transition obeys first-order kinetics.

Relaxivity Values. The factors that determine the values of the relaxivities of Mn^{2+} complexes with Con A are of little concern to the main results of the paper: the discovery of two conformation states of Con A and the determination of the kinetics of the transformation between the two conformers.

TABLE II: Values for τ_M, the Residence Lifetime of a Water Molecule Ligand of Mn^{2+} Bound to Con A at S1, Derived from a Fit of Relaxation Theory (Koenig et al., 1973) to the Data of Figure 2a-b.[a]

Complex	pH	τ_M (μs) 5 °C	τ_M (μs) 25 °C
MP	5.3	0.38	0.20
MP	5.6	0.34	0.19
MP	6.0	0.30	0.17
MP	6.4	0.37	0.21
CMPL	5.3	1.4	1.2
CMPL	5.6	1.5	1.3
CMPL	6.0	1.4	1.2
CMPL	6.4	1.5	1.3

[a] No corrections have been made for the small concentration of Mn^{2+}-Con A ternary complex in some of the MP samples (see text).

What is of importance was the capability of relaxation dispersion data to indicate the concentration of free Mn^{2+}, and to distinguish among different forms of bound Mn^{2+}. Nonetheless, some relevant information may be obtained from the relaxivity values. Meirovitch and Kalb (1972) were the first to report a difference in the relaxivities of apo-Con A with only Mn^{2+}, and with both Mn^{2+} and Ca^{2+}, added. They observed the high-solvent proton relaxivity of MP and the lower relaxivity of CMPL, at 8 and 50 MHz, and also measured the relaxivity of solvent ^{17}O. They estimate an uncertainty of ±10% for their T_1 values; within this uncertainty the agreement with our results is only fair. They concluded that the "mean residence time (τ_M) of the bound water molecule in Mn^{2+}-Con A is approximately 0.1 μs and increases to approximately 1 μs on binding of Ca^{2+}." These results for τ_M agree very well with our results (Table II) derived by the procedures used previously for CMPL by Koenig et al. (1973). However, values of τ_M for MP derived by Barber and Carver (1975) are an order of magnitude shorter than those derived here; we believe the former to be incorrect because of the following. It has been argued that to obtain values of τ_M from relaxation measurements, either data must be obtained in the limit of very low fields (Koenig et al., 1973), or else ancillary experiments such as ^{17}O relaxation measurements must be performed (Koenig and Epstein, 1975). Neither was done by Barber and Carver. Details of these arguments with respect to Con A will be presented elsewhere. Qualitatively, when τ_M becomes comparable to, or longer than, the relaxation time of a proton on a water molecule hydrating an Mn^{2+} ion, the features of the dispersion curve becomes washed out. In the limit of long τ_M, T_1 is simply proportional to τ_M and independent of magnetic field. The changes in relaxation dispersion of MPE and CMPL with temperature (Figures 4a,b) is a result of the variation of τ_M with temperature, and the different relaxivities of these two species results from an order of magnitude change in τ_M when Ca^{2+} binds to MP. We note that the results for τ_M (Table II) (as for the relaxivities and for T_L) are essentially pH independent, particularly for CMPL. For the MP samples, the one at pH 6.0 has the shortest τ_M, and, from Figure 3a,b and other data, the greatest relaxivity. The slightly greater values of τ_M for three other samples is undoubtedly due to the demonstrated presence of a small amount (about 12%) of the ternary complex MMPL.

Concluding Remarks. The major interest in Con A centers around its saccharide-binding and agglutination properties. A fundamental question concerns the relationship between metal-ion binding activity of Con A and its saccharide-binding activity. Most investigations of saccharide-binding and agglutination properties of Con A have been done with forms of the protein which are essentially identical to the stable ternary Ca^{2+}-Mn^{2+}-Con A complex. However, we have shown that binding of Ca^{2+} at S2 ultimately produces protein in a specific conformation, the locked one, which means that most saccharide binding and agglutination experiments have been done with a particular conformation of the protein. The question that arises, then, is whether the saccharide-binding and agglutination properties of Con A require the presence of metals, Ca^{2+} in particular, or really only require the protein to be in the locked conformation. Since we are now able to prepare demetallized protein in the locked conformation, the question takes on new significance. Experiments to clarify these issues are in progress.

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