Conformation as the Determinant of Saccharide Binding in Concanavalin A: Ca^{2+}–Concanavalin A Complexes[†]

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ABSTRACT: The existence of two conformational states of concanavalin A (Con A) with different metal ion binding properties has been recently demonstrated (Brown, R. D., Brewer, C. F., & Koenig, S. H. (1977) *Biochemistry 16*, 3883). Introduction of Mn^{2+} to the S1 site and Ca^{2+} to the S2 site of apo-Con A was shown to induce a conformational change in the protein, ascribed to a cis–trans isomerization of a peptide bond in the secondary structure, which results in extremely tight binding of the metal ions. This induced conformation is referred to as "locked" and the initial conformation as "unlocked". The locked ternary complex is identical with the native protein. In the present paper, we report evidence for the formation of a relatively stable, locked, ternary Ca^{2+}–Con A complex that possesses properties similar to those of native Ca^{2+}–Mn^{2+}–Con A. The experimental technique involves measurement of the magnetic field and time dependence of the nuclear magnetic relaxation rate $(1/T_1)$ of solvent water protons in solutions of Ca^{2+}–Con A, after the addition of Mn^{2+} ions which slowly bind to the protein. The kinetic data can be fit by a model for Ca^{2+} interactions with Con A which indicates that Ca^{2+}, in the absence of Mn^{2+}, can bind at both the S1 and the S2 sites of the protein and, furthermore, can induce the protein to undergo the unlocked to locked conformational transition. In terms of this model, the time-dependent binding of the Mn^{2+} ions is due to replacement of Ca^{2+} ions at the S1 sites in the locked protein. The off-rate of Ca^{2+} from the S2 site of the locked ternary Ca^{2+}–Con A complex is much greater than that from the locked Ca^{2+}–Mn^{2+}–Con A complex. From the effects of added α-methyl D-mannopyranoside on the rate of replacement of Ca^{2+} by Mn^{2+} at the S1 site of the locked ternary Ca^{2+}–Con A complex, it is concluded that the latter complex binds saccharides as strongly as the locked Ca^{2+}–Mn^{2+}–Con A complex. In addition, analysis of the data indicates that apo-Con A in the locked conformation binds α-methyl D-mannopyranoside with approximately 7% of the affinity of the fully metallized locked form of the protein. This strong saccharide-binding activity of locked apo-Con A, compared with that of the unlocked apo-Con A, was further demonstrated by equilibration of unlocked apo-Con A with α-methyl D-mannopyranoside, which resulted in the formation of the locked apo-Con A–saccharide complex. These results demonstrate that it is the locked conformation of Con A that is primarily responsible for saccharide-binding activity, and that the function of the bound metals is primarily to maintain the protein in the locked conformation.

T he utility of concanavalin A (Con A[1]) as a probe for investigating the properties of cell surfaces (cf. Bittinger & Schnebli, 1976) relates directly to its saccharide-binding activity. This activity has been shown to depend on the prior association of certain metal ions with two ion-specific sites per Con A monomer (27 000 daltons): a site S1, the "transition-metal" site, and a site S2, the "calcium" site, that binds Ca^{2+} ions once S1 is occupied (Kalb & Levitzki, 1968).

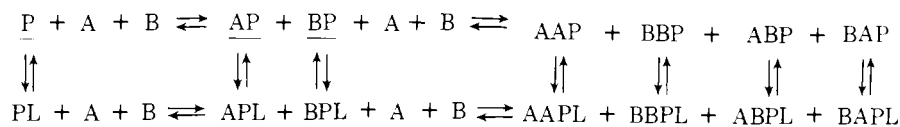Brown et al. (1977) have demonstrated that Con A can exist in two conformations characterized by significantly different metal-binding properties. The conformation of lower energy is the same for both apo-Con A and Con A with S1 occupied by Mn^{2+} ions. This conformation (named the "unlocked" conformation) binds Ca^{2+} at S2 very weakly (dissociation constant ∼ 1 mM at pH 6.4) when Mn^{2+} is bound at S1. Once formed, the unlocked ternary complex undergoes a first-order transition to another conformational state, lower in energy by about 4 kcal M^{-1}. This second conformation binds Ca^{2+} ions very strongly; the off-rate of Ca^{2+} ions from S2 (and hence Mn^{2+} ions from S1) is of order several hours at pH 6 and 25 °C. This conformation (named the "locked" conformation) is that of the native protein. The energy barrier for the unlocked–locked transition is 22 kcal M^{-1} and independent of pH for Mn^{2+} ions at S1 and Ca^{2+} ions at S2.

Brown et al. (1977) also showed that, in the absence of Ca^{2+} ions, Mn^{2+} ions can bind at S2 to form a ternary complex with Con A and induce the unlocked–locked conformational transition, but with a much slower time course than for the Ca^{2+}–Mn^{2+} case, due both to weaker binding of Mn^{2+} ions at S2 and a higher energy barrier for the transition. In addition, it was found that in the locked conformation Mn^{2+} ions do not bind to S2 as tightly as do Ca^{2+} ions. Addition of EDTA to the Mn^{2+}–Con A locked ternary complex at 5 °C resulted in rapid removal of all Mn^{2+} ions from both S1 and S2, producing apo-Con A in a metastable, locked conformation which, in a time of the order of days at 5 °C, reverted to unlocked apo-Con A. The above studies demonstrated that occupation of S2 by metal ions is associated with the induction of a change of conformation of the metal–protein complex to that of the na-

[1] Abbreviations used: Con A, concanavalin A with unspecified metal content; P, AP, and BAP, for, respectively, apo-Con A, the binary complex of Con A with metal-ion A at binding site S1, and the ternary complex of Con A with metal ion B at binding site S2 and A at S1, all with the protein in the "unlocked" conformation; the suffix L (e.g., BAPL) indicates the analogous molecule in the "locked" conformation; the prefix Sa (e.g., SaBAPL) indicates bound saccharide; EDTA, ethylenediaminetetraacetic acid; α-MDM, α-methyl D-mannopyranoside; α-MDGal, α-methyl D-galactopyranoside; NMRD, nuclear magnetic relaxation dispersion.

SCHEME I

$$P + A + B \rightleftharpoons \underline{AP} + \underline{BP} + A + B \rightleftharpoons AAP + BBP + ABP + BAP$$

$$PL + A + B \rightleftharpoons APL + BPL + A + B \rightleftharpoons \underline{AAPL} + \underline{BBPL} + \underline{ABPL} + \underline{BAPL}$$

tive protein (with the attendant strong saccharide binding), and that Mn^{2+} ions as well as Ca^{2+} ions at S2 can serve this function. It is now possible to investigate the effect of the conformation of Con A on its saccharide-binding activity.

Richardson & Behnke (1976) studied the precipitation of polysaccharides by various mixtures of apo-Con A and metal ions and showed that addition of only Mn^{2+} ions or only Ca^{2+} ions to apo-Con A gives rise to precipitation reactions after a substantial time delay. The immediate inference, from the work of Brown et al. (1977), is that the locked ternary Mn^{2+}-Con A complex binds saccharides, and that Ca^{2+} ions alone can also form a ternary complex with Con A, inducing a conformation change to the locked, saccharide-binding form.

In the light of the above observations, we initiated a survey of ternary complexes of Con A containing a single species of metal ion, and of their interactions with saccharide. Our initial goals were to obtain values for the equilibrium constants of the ions in the various complexes, to measure the kinetics of metal binding and release, to establish the conditions that induce conformation change in the protein, and to determine the saccharide-binding activity of the various metal ion complexes. In the present paper, we develop a generalization of the equilibrium schemes discussed by Brown et al. (1977) for interpreting the interactions of Mn^{2+} and Ca^{2+} ions with Con A in the absence of saccharide. The generalization includes competition between more than one type of ion for both S1 and S2, and saccharide binding to Con A in the locked conformation irrespective of the occupation of S1 and S2. We limit the considerations to interactions of Ca^{2+} ions and saccharide with Con A in terms of the generalized scheme. We monitor these interactions by following the competitive binding of Mn^{2+} ions, which we can observe, with Ca^{2+}. In the course of these experiments, we discovered that locked apo-Con A has a saccharide affinity comparable to that of native Con A. Because of the signficance of this finding, which indicates that conformation is the main determinant of saccharide binding, we have organized the presentation of the data and discussion to emphasize this point. In subsequent papers, we will discuss complexes of Con A with other ions, e.g., only Mn^{2+} ions or only Cd^{2+} ions, and the interactions of these complexes with saccharide.

Schemes for Metal–Protein Interactions

There is a plethora of equilibrium and time-dependent phenomena that can be observed when apo-Con A is mixed with metal ions and saccharides in varying concentrations. We have previously determined many of the equilibria and associated pathways for interactions of Mn^{2+} and Ca^{2+} ions with apo-Con A (Brown et al., 1977). In those experiments, conditions were chosen such that it was unnecessary to consider competition between Mn^{2+} and Ca^{2+} ion for the same S1 site. The need now is to generalize the scheme developed for interpreting the interactions of Mn^{2+} and Ca^{2+} ions with Con A in order to include interactions with other ions, competition between different species of ions for the same binding sites, and interactions of the various metal ion Con A complexes with saccharides. This permits organization of the data in a systematic manner, as well as the design of experiments using the

generalized scheme as a working hypothesis. The present experiments have been designed to test various limiting, simplifying cases of the schemes of multiple equilibria (described below), and all our data will be interpreted in terms of them. It is to be realized, of course, that the fact that all data can be described by an equilibrium scheme is not proof of the uniqueness of the scheme. However, the schemes as developed will be no more complex than necessary to explain all the data.

Scheme I shows the proposed multiple equilibria among two types of metal ions (A and B) and apo-Con A, in the absence of saccharide. (This is a generalization of Scheme I of Brown et al. (1977).) Sequential binding of metal ions is assumed for both conformations of the protein; i.e., it is assumed that occupation of the S2 site requires the prior occupation of S1. (Scheme I also mandates the converse: an ion cannot leave S1 while S2 is occupied.) The notation is designed to convey the sequence of the binding: the letter immediately left of P (for protein) denotes the ion in S1; the letter left of that, the ion in S2. The suffix L indicates the locked conformation, its absence, the unlocked conformation. The complexes underlined are those with the lower energy, as anticipated from previous results (Brown et al., 1977) as well as from the present work.

Scheme I simplifies signficantly in certain special cases. If A and B represent Mn^{2+} and Ca^{2+}, respectively, then competition of Ca^{2+} with Mn^{2+} for S1, and Mn^{2+} with Ca^{2+} for S2, may be neglected and the pathway for equilibrium when both metal ions are added at the same time to apo-Con A (produced by the usual demetallization procedures) is P → MnP → CaMnP → CaMnPL. The equilibrium is totally in the direction of CaMnPL, with the last step requiring a conformation change that is first order and has a time-constant of 17 min at 5 °C.

Other simplifying conditions occur when A and B represent identical ions. Scheme I has been shown to be adequate to represent the multiple equilibria and kinetic pathways observed when A and B both represent Mn^{2+} (Brown et al., 1977). The special case of A and B both representing Ca^{2+} is the subject of the present paper. Scheme I then simplifies to Scheme II. The generalization of Scheme II to include saccharide binding is considered below.

SCHEME II

$$P + Ca \rightleftharpoons CaP + Ca \rightleftharpoons CaCaP$$

$$PL + Ca \rightleftharpoons CaPL + Ca \rightleftharpoons CaCaPL$$
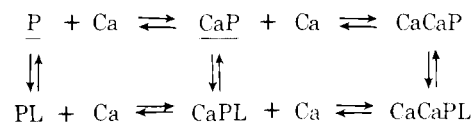
Materials and Methods

Materials. Apo-Con A was prepared by demetallizing the protein at low pH as previously described (Brown et al., 1977). All samples were prepared in pH 6.4, 0.1 M potassium acetate, 0.9 M KCl buffer. The protein concentration was approximately 0.85 mM monomer in all cases, as determined spectrophotometrically at pH 5.6 using an absorbance $A_{280nm}^{1\%,1cm}$ = 12.4 (Yariv et al., 1968; Brown et al., 1977), and a molecular weight of 27 000 per monomer. All protein concentrations are expressed in monomeric units.

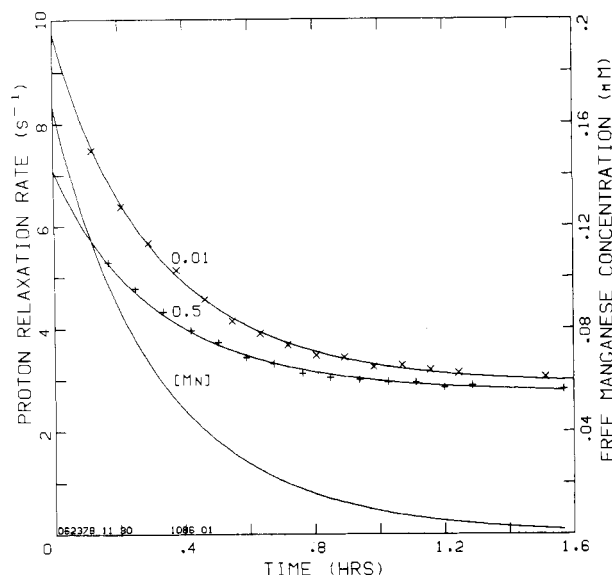Stock solutions of 0.1 M $MnCl_2$ and $CaCl_2$ were standard-

FIGURE 1: Solvent proton relaxation rate data at 0.01 and 0.5 MHz (2.2 and 110 Oe), given by X and +, respectively, as a function of time at 5 °C, after addition of 0.16 mM Mn^{2+} to 0.85 mM apo-Con A that had been equilibrated for about 2 h at 25 °C with 10 mM Ca^{2+}. The sample was cooled to 5 °C before the addition of Mn^{2+} ions. The solid lines through the data points result from a least-squares comparison of the data with eq 5 and 8; the remaining solid shows the corresponding time dependence of the concentration of free Mn^{2+} ions.

ized by EDTA titration. Metals were introduced by adding microliter amounts of these stock solutions directly to 0.6 mL of the apo-Con A solution. The saccharide used in most cases was α-methyl D-mannopyranoside (α-MDM), obtained commercially; α-methyl D-galactopyranoside (α-MDGal), which binds much more weakly to native Con A than α-MDM, was used as a control.

Methods. The concentrations of both free and bound Mn^{2+} ions in the samples were monitored by the technique of NMRD, in which the spin–lattice relaxation rate $(1/T_1)$ of solvent protons is measured as a function of magnetic field between 2 Oe and 12 kOe.[2] The resultant variation (called a dispersion curve) has an identifiable contribution from free Mn^{2+} ions, as well as contributions from bound ions that depend specifically on the complex. The NMRD procedures used are identical with those used by Brown et al. (1977), and further details and other references are found therein.

Results

Interactions in the Absence of Saccharide. Figure 1 shows solvent proton relaxation rates at 0.01 and 0.5 MHz (2.2 and 110 Oe) as a function of time at 5 °C after addition of 0.16 mM Mn^{2+} to a 0.85 mM apo-Con A sample that had been equilibrated at 25 °C for about 2 h with 10 mM Ca^{2+} and then cooled. As indicated previously (cf. Brown et al., 1977, Figure 1), the difference in relaxation rates between 0.01 and 0.5 MHz is directly proportional to the concentration of free Mn^{2+} (except for a small correction for the apoprotein). The lines through the data points are from a least-squares comparison of the data with Scheme III (below), using the theoretical analysis presented in the Theory and Data Analysis section. The derived time dependence of the free Mn^{2+} concentration is shown by the remaining solid line. It is seen that all the added Mn^{2+} is initially free, and that the Mn^{2+} ions all become bound
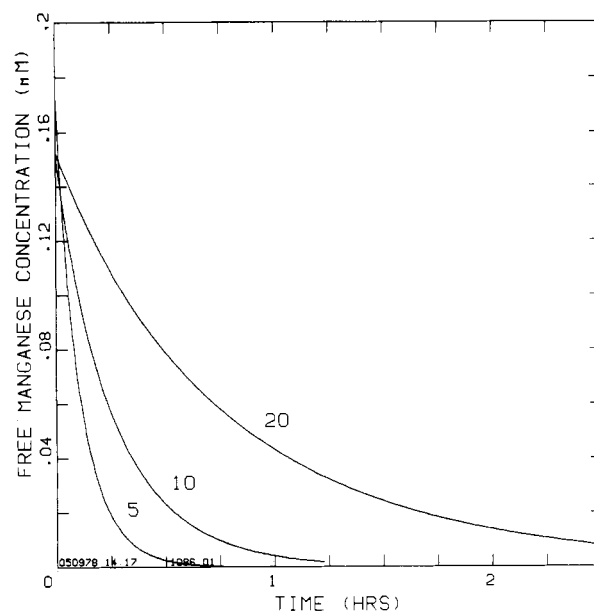
FIGURE 2: The time dependence of the concentration of free Mn^{2+} ions at 5 °C derived from data similar to those of Figure 1, for samples of apo-Con A equilibrated with differing total Ca^{2+} concentrations (nominally 5, 10, and 20 mM, as indicated) at 25 °C, before the addition of 0.16 mM Mn^{2+} at 5 °C.

at long times. Figure 2 shows results for the time dependence of the free Mn^{2+} concentration, derived from experiments similar to that of Figure 1, but with differing values for the total Ca^{2+} ion content. Qualitatively, it is seen that the time course is slowed for the samples with increasing Ca^{2+} ion concentration. Similar experiments were performed for samples at 25 °C. A quantitative analysis of all the data will be given in the Discussion section. Several controls were also run in which Mn^{2+} ions were added 2 days rather than 2 h after the Ca^{2+} addition. The results were the same.

Interactions in the Presence of Saccharide. Experimental results for a sample of apo-Con A equilibrated with 10 mM Ca^{2+} at 25 °C, after which 10 mM α-MDM and 0.16 mM Mn^{2+} were added at 5 °C, are shown in Figure 3. Addition of α-MDM either before or after the equilibration with Ca^{2+} ions gave identical results. Fits of the data with Scheme IV (discussed below), a generalization of Scheme II that includes saccharide-binding, give the solid lines in Figure 3. A comparison of this figure with Figure 1 shows that the presence of 10 mM α-MDM increases the time scale about tenfold.

Figure 4 shows results for the time dependence of the free Mn^{2+} concentration at 5 °C derived from experiments similar to that of Figure 3, for various concentrations of α-MDM and total Ca^{2+}. Two qualitative results should be noted: as the saccharide concentration is increased, the time course of the decay of the free Mn^{2+} concentration increases about tenfold and then it saturates. Similar results were obtained at 25 °C and are included in the theoretical analysis below.

Finally, as a control, an experiment similar to that of Figure 3, but at 25 °C, and with 0.1 mM α-MDGal as the added saccharide was performed. The effect on the time course was about one-tenth that produced by the same concentration of α-MDM.

Interactions of α-MDM with Apo-Con A. A sample of apo-Con A was equilibrated with 1.1 M α-MDM for 11 days at room temperature. Subsequent addition of 5 mM Ca^{2+} and 0.33 mM Mn^{2+} at 5 °C resulted in the formation of the complex of CaMnPL and α-MDM in less than 15 min, as evidenced from the observed relaxivity dispersion. In a control
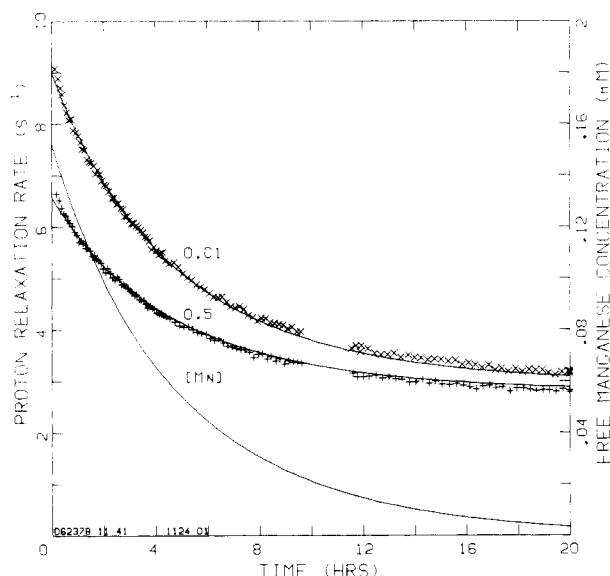
FIGURE 3: Solvent protein relaxation rate data at 0.01 and 0.5 MHz (2.2 and 110 Oe), given by X and +, respectively, as a function of time at 5 °C, after addition of 0.16 mM Mn^{2+} and 10 mM α-MDM to 0.85 mM apo-Con A that had been equilibrated for about 2 h at 25 °C with 10 mM Ca^{2+}. The sample was cooled to 5 °C before the additions of Mn^{2+} and saccharide. The solid lines through the data points result from a least-squares comparison of the data with eq 5 and 8; the remaining solid line shows the corresponding time dependence of the concentration of free Mn^{2+} ions.
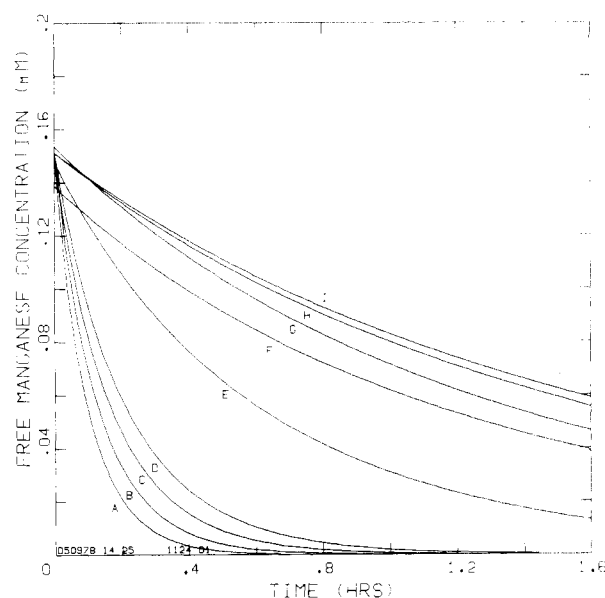

FIGURE 4: The time dependence of the concentration of free Mn^{2+} ions at 5 °C derived from data similar to those in Figure 3, for samples of apo-Con A equilibrated with Ca^{2+} at 25 °C, before the addition of 0.16 mM and varying amounts of α-MDM at 5 °C. Curves A, C, E, F, G, and H are for samples which contained 10 mM total Ca^{2+} concentration and, respectively, 0, 0.5, 2.5, 10, 100, and 250 mM α-MDM. Curves B, D, and I correspond to 8 mM total Ca^{2+} and 0.5, 1, and 500 mM α-MDM.

sample, to which α-MDM was added at 5 °C immediately before the Ca^{2+} and Mn^{2+}, the time course for complete formation of CaMnPL was of the order of a day.

Ancillary Experiments. In order to interpret the above data, several ancillary experiments were necessary to measure the effect of the increase in solvent viscosity, due to the presence of α-MDM, on the relaxivities of free Mn^{2+} ions, apo-Con A, and the relaxation rate of the buffer. We have obtained complete dispersion curves for solvent proton relaxation due to Mn^{2+} aquoions for several concentrations of α-MDM. The derived values of relaxivity at 0.01 and 0.5 MHz are given in Table I, as are the changes in the relaxation rates of the buffer. The effects on the relaxivity of free Mn^{2+} are significant and can be understood in terms of the known mechanisms of relaxation of protons by Mn^{2+} aquoions as influenced by viscosity changes (cf. Koenig et al., 1971; Rubinstein et al., 1971). The effect of saccharide on the relaxivity values of apo-Con A and CaCaPL, found to be the same, is also large; 1 M α-MDM increases the low-field relaxivity by a factor of about two and lowers the inflection frequency of the dispersion by about the same amount. Again, these changes appear to be due to viscosity effects (cf. Hallenga & Koenig, 1976).

During the course of the experiments several qualitative observations on the stability of Con A solutions under various conditions were noted. Our usual procedure is to store apo-Con A solutions (typically, 0.5 to 1.0 mM) at room temperature to minimize small drifts in the NMRD of Mn–Con A samples observed when the storage temperature was 5 °C (Brown et al., 1977). For either storage temperature, upon addition of Ca^{2+}, the resulting CaMnPL gave identical dispersion results. With storage of apo-Con A solutions at 25 °C, there is a gradual precipitation; however, over a period of weeks there are no apparent changes in the properties of the remaining dissolved protein. Solutions of SaCaMnPL appear to be the most stable, remaining clear for many weeks. CaMnPL is somewhat more stable than the apoprotein. However, for samples equilibrated with only Ca^{2+}, although the solutions remained clear, significant crystallization of protein was ob-

TABLE I: The Effect of 500 mM α-MDM on the Relaxivity Values of Solvent Protons, for Two Values of Magnetic Field, in Solutions of Mn^{2+} Aquoions (R_M), apo-Con A (R_P), and Buffer $(1/T_{1w})$.[a]

T (°C)	[α-MDM] (mM)	field (MHz)	R_M (mM s)$^{-1}$	R_P (mM s)$^{-1}$	$1/T_{1w}$ (s^{-1})
5	0	0.01	49.6	0.99	0.57
5	500	0.01	49.8	1.17	0.74
5	0	0.5	32.2	0.74	0.57
5	500	0.5	37.2	0.99	0.74
25	0	0.01	46.7	0.62	0.40
25	500	0.01	42.7	0.71	0.50
25	0	0.5	19.8	0.48	0.40
25	500	0.5	21.3	0.55	0.50

[a] These values are required when using eq 8. Data obtained at several values of [α-MDM] indicated that the variation of relaxivity values was linear with saccharide concentration up to 500 mM.

served over periods of several days. Microscopic examination of the crystals showed them to have the same morphology as the native protein (Hardman, private communication).
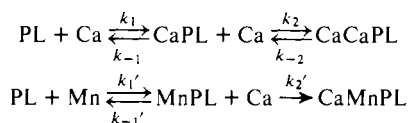
For the samples in Figure 2, and others, additional increments of Mn^{2+} ions were added after the initial run, and the time course of the binding of the added Mn^{2+} followed. The time course of Mn^{2+} binding (corrected for initial conditions as discussed in the schemes below) lengthened by a factor of 2 when the total Mn^{2+} added became close to the total protein concentration.

Theory and Data Analysis

Interactions in the Absence of Saccharide. All the data obtained in the absence of saccharide can be fit by a simple kinetic model derived directly from Schemes I and II. In view of the fact that Mn^{2+} ions were added to all samples (except the controls) only after equilibration of the apoprotein with Ca^{2+} ions for at least 2 h, and as much as 2 days, at room

temperature, with no difference in the results, we assume that the protein molecules have all taken on the locked conformation, mainly CaCaPL, before the addition of Mn^{2+} ions. (In reality, an equilibrium between populations of locked and unlocked conformations has been reached, but we will show from the data that the equilibrium is essentially all in the locked direction, with Ca^{2+} ions at both S1 and S2 of every monomer.) With this assumption, it becomes straightforward to write an expression for the time dependence of [Mn], the concentration of free Mn^{2+} ions, in terms of the rate constants and equilibrium constants that describe the equilibria among the several locked complexes. Scheme III displays the kinetic pathways involved.

SCHEME III

$$PL + Ca \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CaPL + Ca \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} CaCaPL$$

$$PL + Mn \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} MnPL + Ca \overset{k_2'}{\rightarrow} CaMnPL$$

It is assumed that the formation of CaMnPL is responsible for the disappearance of Mn^{2+} ions, and that the final step MnPL → CaMnPL may be regarded as irreversible. The disappearance rate of free Mn^{2+} is then proportional to the rate of formation of CaMnPL:

$$\frac{d[Mn]}{dt} = -\frac{d[CaMnPL]}{dt} = -k_2'[Ca][MnPL] \quad (1a)$$

or, in terms of [CaCaPL]:

$$\frac{d[Mn]}{dt} = -k_{-2}\frac{[Mn]K_{CPL}}{[Ca]K_{MPL}}\frac{k_2'}{k_2}[CaCaPL] \quad (1b)$$

where

$$K_{CPL} = k_{-1}/k_1 = [Ca][PL]/[CaPL] \quad (1c)$$

$$K_{CCPL} = k_{-2}/k_2 = [Ca][CaPL]/[CaCaPL] \quad (1d)$$

$$K_{MPL} = k_{-1}'/k_1' = [Mn][PL]/[MnPL] \quad (1e)$$

and

$$[CaCaPL] = [P_T] - [Mn_T] + [Mn] \quad (2)$$

$[P_T]$ and $[Mn_T]$ are the total protein monomer and Mn^{2+} ion concentrations, respectively. The approximations are made that the amounts of Mn^{2+} in the MnPL complex and Ca^{2+} in the CaPL complex are at all times small compared with their respective totals, and that $[P_T] \simeq [CaCaPL] + [CaMnPL]$; i.e., [P], [MnPL], and [CaPL] are negligible. Also, it is assumed that the velocity along the irreversible pathway is low in the sense that all relative concentrations other than [CaMnPL] are determined by their respective equilibrium constants; in the experiments, this condition was assured by keeping [Ca] ≫ [Mn].

Equation 1b shows that the rate of disappearance of [Mn] is equal to [CaCaPL] times k_{-2}, the off-rate of Ca^{2+} from this complex, multiplied by corrections for the relative competitive rates of reformation of the ternary complexes.

For our experimental conditions, the total concentration of $[Ca^{2+}]$ is sufficiently greater than the amount replaced by Mn^{2+} ions so that [Ca] in eq 1b may be considered constant in time; we take it equal to the total less the time average of that bound. It is then straightforward to integrate eq 1b. Combining eq 1b and 2 gives

$$\frac{d[Mn]}{dt} = -\frac{[Mn]}{\tau}\left(1 + \frac{[Mn]}{[P_T] - [Mn_T]}\right) \quad (3)$$

where

$$\tau = \frac{k_2 K_{MPL}}{k_{-2}k_2'K_{CPL}}\frac{[Ca]}{[P_T] - [Mn_T]} \equiv \tau_R \frac{[Ca]}{[P_T] - [Mn_T]} \quad (4)$$

Integrating eq 3, one obtains the following expression for [Mn] as a function of time:

$$[Mn] = \frac{([P_T] - [Mn_T])[Mn_T]}{([P_T]\exp[(t - t_0)/\tau]) - [Mn_T]} \quad (5)$$

Here τ_R is a time constant that depends only on the rate constants in Scheme III and is always positive. τ, however, depends on the concentrations of ions and protein, and its sign depends on the sign of $[P_T] - [Mn_T]$. The experimentally observed boundary condition, that $[Mn] = [Mn_T]$ at the initial time $t = t_0$, has been imposed to obtain eq 5.

The right hand side of eq 5 can be expanded to give the initial decay-time of [Mn] for short times, i.e., for $(t - t_0) \ll \tau$, which we define as τ_1:

$$[Mn] \simeq [Mn_T]\left(1 - \frac{[P_T]}{([P_T] - [Mn_T])}\frac{(t - t_0)}{\tau}\right)$$
$$= [Mn_T](1 - (t - t_0)/\tau_1) \quad (6)$$

where

$$\tau_1 = \tau_R[Ca]/[P_T] \quad (7)$$

Equation 7 is particularly useful as a check on the somewhat complex fitting procedures since it directly relates τ_R, which is independent of the concentrations of the components of the sample, to the observed initial behavior in any particular situation.

To compare this theory with the experimental relaxation rates so as to derive values for τ, we require an expression that relates the observed relaxation rate $1/T_1$ at a given magnetic field to [Mn], in terms of the concentrations of the components of the samples and their respective relaxivities:

$$1/T_1 = R_M[Mn] + R_{CMPL}([Mn_T] - [Mn])$$
$$+ R_P[P_T] + 1/T_{1w} \quad (8)$$

Here R_M is the relaxivity of the Mn^{2+} aquoion; R_{CMPL} is the relaxivity of the bound Mn^{2+} complex CaMnPL; R_P is the relaxivity of the diamagnetic protein (small and observed to be the same for apo-Con A and the Ca^{2+}-Con A ternary complex); and $1/T_{1w}$ is the relaxation rate of the buffer solution. These quantities, which depend on temperature, magnetic field, and concentration of saccharide, were determined independently of the time-dependent data from the various ancillary experiments described.

The solid lines in Figures 1 and 2 are derived from a least-squares comparison of the data with eq 5 and 8. The parameters τ and t_0 are treated as unknown parameters in all cases, with one set of values to be found that fits the 0.01 and 0.5 MHz data simultaneously. The relaxivities at 0.01 and 0.5 MHz for CaMnPL are constrained to be equal. This assures the fit for $t \rightarrow \infty$. The results for τ and τ_R are given in Table II. The parameter t_0 is also known reasonably well, but in several cases the data are changing so rapidly at short times that it is better to derive a value for t_0 than to consider it known initially, given that the time for a measurement of $1/T_1$ can be comparable to the value of τ_1.

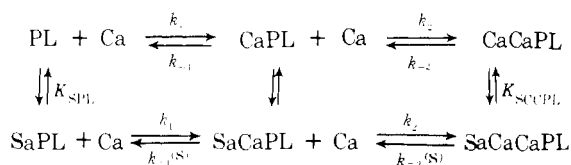*Interactions in the Presence of Saccharide.* In order to interpret the data on Ca^{2+} replacement in the presence of saccharide (Figures 3 and 4), Scheme III must be augmented to allow for binding of saccharide to the various complexes in the locked conformation. From the traditional view of sequential binding, one might infer that saccharide binds only to the ternary metal-Con A complexes. However, the data cannot

TABLE II: Results for τ, $\tau^{(S)}$, τ_R, and $\tau_R^{(S)}$, for All Samples Considered, Including Those for Which Data Are Shown in the Figures.[a]
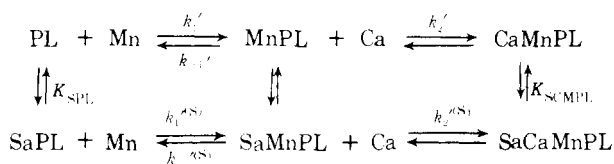
T (°C)	$[Ca^{2+}]^b$ (mM)	$[\alpha\text{-MDM}]^b$ (mM)	τ, $\tau^{(S)}$ (h)	τ_R, $\tau_R^{(S)}$ (h)
5	5	0	0.13	0.024
	10	0	0.37	0.028
	10	0	0.37	0.028
	10	0	0.32	0.026
	10	0	0.29	0.024
	20	0	0.88	0.030
	20	0	0.89	0.031
25	10	0	0.14	0.011
	10	0	0.11	0.009
	20	0	0.31	0.012
5	10	0.50	0.49	0.040
	10	2.5	3.1	0.26
	10	10.0	5.5	0.46
	10	100	5.2	0.43
	10	500	4.9	0.40
25	8	0.50	0.15	0.015
	10	0.50	0.19	0.016
	8	1.0	0.24	0.025
	10	2.5	0.72	0.059
	10	10.0	0.40	0.12
	10	100	2.0	0.16
	10	250	1.8	0.15
	10	500	1.5	0.16
	10	100 α-MDGal[c]	0.9	0.016

[a] In all cases, Ca^{2+} ions in the total concentrations indicated were added to 0.85 mM apo-Con A (nominal monomer concentration) and the samples equilibrated for at least 2 h at 25 °C. Mn^{2+} ions and saccharide were added subsequently, with the samples at the temperature at which data were taken. Results for τ and $\tau^{(S)}$, which come directly from a least-squares comparison of data and theory, depend on the concentrations of the constituents of the samples as well as on the rate parameters of the model schemes. The values for τ_R and $\tau_R^{(S)}$, however, depend only on rate constants and equilibrium constants in Scheme IV; in terms of this scheme, τ_R and $\tau_R^{(S)}$ are, approximately, reciprocals of the off-rates of Ca^{2+} ions from S2 of the complexes CaCaPL and SaCaCaPL, respectively. [b] These are nominal values of concentration, uncorrected for small dilution effects which are considered in obtaining the final results. [c] α-MDGal, a saccharide that binds weakly to native Con A, was used as a control.

be fit by so restrictive a scheme. Therefore we generalize Scheme III to Scheme IV by allowing saccharide to bind to all

SCHEME IV

$$PL + Ca \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CaPL + Ca \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} CaCaPL$$

$$\Big\updownarrow K_{SPL} \qquad \Big\updownarrow \qquad \Big\updownarrow K_{SCCPL}$$

$$SaPL + Ca \underset{k_{-1}^{(S)}}{\overset{k_1}{\rightleftharpoons}} SaCaPL + Ca \underset{k_{-2}^{(S)}}{\overset{k_2}{\rightleftharpoons}} SaCaCaPL$$

and

$$PL + Mn \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} MnPL + Ca \underset{}{\overset{k_2'}{\rightleftharpoons}} CaMnPL$$

$$\Big\updownarrow K_{SPL} \qquad \Big\updownarrow \qquad \Big\updownarrow K_{SCMPL}$$

$$SaPL + Mn \underset{k_{-1}^{(S)}}{\overset{k_1^{(S)}}{\rightleftharpoons}} SaMnPL + Ca \overset{k_2^{(S)}}{\rightleftharpoons} SaCaMnPL$$

locked forms, PL, CPL, CCPL, MPL, and CMPL, and define the following additional dissociation constants:

$$K_{SPL} = [Sa][PL]/[SaPL] \qquad (9a)$$

$$K_{SCCPL} = [Sa][CaCaPL]/[SaCaCaPL] \qquad (9b)$$

$$K_{MSPL} = [Mn][SaPL]/[SaMnPL] \qquad (9c)$$

We can neglect the formation of MnMnPL in the presence of Ca^{2+}.

Allowing Mn^{2+} ions to bind to both PL and SaPL and follow the two indicated parallel irreversible pathways in Scheme IV, one can derive a rate equation analogous to eq 1b. The result is (using eq 2)

$$\frac{d[Mn]}{dt} = -k_{-2}\frac{[Mn]}{[Ca]}\frac{K_{CPL}}{K_{MPL}}\frac{k_2'}{k_2}([P_T] - [M_T] + [Mn])G \qquad (10a)$$

where

$$G = \frac{1 + [Sa]/(K_{SPL}(K_{MSPL}k_2'/K_{MPL}k_2'^{(S)}))}{1 + [Sa]/K_{SCCPL}} \qquad (10b)$$

This is the same functional form as eq 1b and can be integrated to give, with the same assumptions as before, results identical with eq 3, 4, and 5, with τ and τ_R replaced respectively by $\tau^{(S)}$ and $\tau_R^{(S)}$, where

$$\tau_R^{(S)} = \tau_R/G \qquad (11)$$

The manner in which $\tau_R^{(S)}$ deviates from τ_R as saccharide is added can be seen by referring to G, eq 10b. If, for example, saccharide does not bind to PL, i.e., if K_{SPL} is infinite, then as $[Sa] \rightarrow \infty$, $G \rightarrow 0$, and $\tau_R^{(S)}$ would increase without limit as the saccharide concentration increased. (The same situation would hold if the pathway containing saccharide complexes were disallowed; i.e., if $k_2'^{(S)} = 0$.) Conversely, if $\tau_R^{(S)}$ does saturate as $[Sa]$ increases, then saccharide *must* bind to PL, i.e., to apo-Con A in the locked conformation, in addition to binding to CaCaPL. The conditions that $\tau_R^{(S)}$ and $1/\tau_R^{(S)}$ respectively attain the mean values between their $[Sa] = 0$ and $[Sa] = \infty$ limits are, from eq 10b

$$[S] = K_{SPL}(K_{MSPL}k_2'/K_{MPL}k_2'^{(S)}) \qquad (12a)$$

and

$$[S] = K_{SCCPL} \qquad (12b)$$

Finally, the ratio of the two limiting values is

$$\tau^{(\infty)}/\tau_R^{(0)} = k_2'^{(S)}K_{MPL}K_{SCCPL}/k_2'K_{MSPL}K_{SPL}$$

$$\simeq K_{SCCPL}/K_{SPL} \qquad (13)$$

if the binding of saccharide to PL does not influence the binding of Mn^{2+} very much and does not significantly alter the rate of the irreversible step.

Values for $\tau_R^{(S)}$ were obtained by the same least-squares procedure as for the saccharide-free data, as were the time dependences of [Mn] and $1/T_1$. The results give the solid lines in Figures 3 and 4; the derived values of $\tau^{(S)}$ and $\tau_R^{(S)}$, at both 5 and 25 °C, are presented in Table II. In Figure 5, the derived values of $\tau_R^{(S)}$ are plotted as a function of α-MDM concentration, for 5 and 25 °C. The curves through the data points are a fit to eq 11, using the values 0.07 and 0.24 mM for K_{SCCPL} at 5 and 25 °C, respectively, and the values 1.2 and 3.9 mM for $K_{SPL}(K_{MSPL}k_2'/K_{MPL}k_2'^{(S)})$. The necessary relation between [Sa] and total saccharide concentration was obtained assuming both [SaPL] and [SaMPL] much smaller than the concentration of the quaternary saccharide-metal-Con A complexes. Also indicated in the figure is one control point, using α-MDGal, a saccharide known to bind weakly to native Con A.

The unassailable conclusion is that α-MDM binds to both PL and CaCaPL, though about 15-fold more weakly to the demetallized form.

Discussion

Understanding the origin of the saccharide-binding activity of Con A and the role of metal ions in mediating this function is of fundamental importance. We have previously established (Brown et al., 1977) that binding of Ca^{2+} at S2 to the equilibrium Mn^{2+}-Con A binary complex initiates a conformation change to the native, active, form of Con A. This conformational transition was also observed to occur for the ternary Mn^{2+}-Con A complex. The obvious question arising from these studies was whether the locked conformation of Con A alone is adequate for strong saccharide binding, or whether metal ions do more than simply maintain the protein in the locked conformation. Motivated by these previous observations, we initiated studies of various ternary complexes of Con A containing a single species of metal-ion, and of their interactions with saccharide.

In the present paper, we report on the interactions of Ca^{2+} ions and saccharide with Con A. The results provide strong evidence that, in contrast to early indications (cf. Kalb & Levitzki, 1968), Ca^{2+} can bind at both the S1 and S2 sites of apo-Con A and induce the conformational transition leading to the locked, active form of the protein. Though the off-rate of Ca^{2+} from the S2 site of CaCaPL is much greater than from CaMnPL, the saccharide-binding activities of both are essentially equal. Additional experiments indicate that PL also binds α-MDM, with approximately 7% of the affinity of the fully metallized locked conformation of Con A, which is at least a factor 10^3 greater than its binding to P (Koenig et al., to be published). These results and others show that it is the locked conformation of Con A that is primarily responsible for the saccharide-binding activity of the protein, and that the function of the bound metals is to maintain the locked conformation. The detailed considerations follow.

Interactions of Ca^{2+} Ions with Apo-Con A. There are several indications in the literature that the addition of only Ca^{2+} ions to Con A is adequate to produce a Con A complex that, after a sufficient time delay, either interacts with saccharide, or interacts with Mn^{2+} in an altered fashion. Richardson & Behnke (1976) showed that 1 mM Ca^{2+} added to apo-Con A will precipitate yeast mannins after about 30 h at room temperature (and pH near 5). Ganz (private communication) has repeated these results with dextrans and 10 mM concentrations of Ca^{2+} ions and finds that about 1 h is adequate to initiate substantial precipitation. Grimalde & Sykes (1975) observed effects on Mn^{2+} binding after equilibration of apo-Con A with Ca^{2+} ions, which they attributed to the preequilibration. We have argued (Brown et al., 1977) that this could also be explained by Mn^{2+} ions binding at both S1 and S2; however, an indication of a Ca^{2+}-Con A complex may be there. Recently, Alter et al. (1977) found that binding of Mn^{2+} to Ca^{2+}-Con A mixtures is altered by preequilibration with Ca^{2+} and showed directly that Ca^{2+} ions bind to Con A in the absence of other metal ions.

In the present work, we have shown that the time dependence of the binding of Mn^{2+} ions to apo-Con A that has been equilibrated with Ca^{2+} ions can be described by Scheme III. Ca^{2+} ions bind to the protein to form CaCaPL which, upon addition of Mn^{2+} ions, undergoes a conversion to CaMnPL; i.e., Mn^{2+} replaces Ca^{2+} in the S1 sites of the locked protein. The time dependence of the replacement process is described by the time constants τ and τ_R, derived in eq 3–5, which show that the time course for binding of Mn^{2+} depends both on the
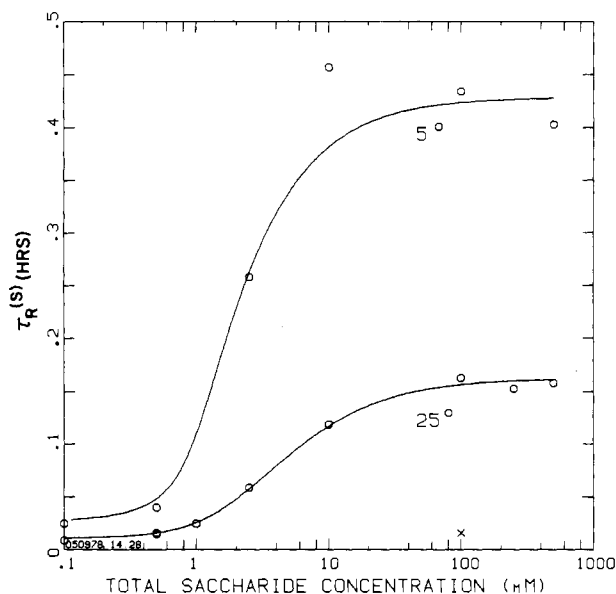

FIGURE 5: Variation of $\tau_R^{(S)}$ (eq 5 and 11) with total saccharide concentration at 5 and 25 °C. The circles indicate the results for α-MDM, and the cross the result for the control, α-MDGal. The solid lines through the data points derive from eq 11, with values for K_{SCCPL} of 0.070 and 0.24 mM and values for $K_{SPL}(K_{SMPL}k_2'/K_{MPL}k_2'^{(S)})$ of 1.2 and 3.9 mM, at 5 and 25 °C, respectively.

rate constants in Scheme III and on the concentrations of metal ions and protein (Table II). These results provide direct evidence that Ca^{2+} alone is sufficient to induce the protein to undergo the unlocked to locked conformational transition discovered by Brown et al. (1977).

We have not as yet measured, quantitatively, the kinetics of the transition between the unlocked and locked Ca^{2+}-Con A complexes, though we know it to be of the order of several minutes at 25 °C, which is somewhat slower than the transition CaMnP $\rightarrow$ CaMnPL, and faster than the MnMnP $\rightarrow$ MnMnPL transition (of the order of days). Harrington & Wilkins (1978) have reported similar estimates. Thus the energy barrier for the unlocked–locked transition depends upon the types of ions bound at both S1 and S2. It remains to be considered whether, in our samples, all Con A molecules are locked by the Ca^{2+} ions at the concentrations of total Ca^{2+} used, and whether Ca^{2+} ions are at both S1 and S2 (i.e., whether the transition is between ternary complexes), and whether the equilibrium represented by the lower line of Scheme II (and upper line of Scheme III) is essentially all to the right. (It tends not to be, for example, if Mn^{2+} rather than Ca^{2+} is the only metal ion present.) The latter is straightforward to argue; any free PL present would immediately (i.e., in less than about 1 min) take up stoichiometric amounts of added Mn^{2+} ions in the presence of the excess Ca^{2+} to form CaMnPL (Brown et al., 1977). However, the data show that all added Mn^{2+} is initially free. Thus, no observable PL is present. Similarly, if the conformation change is not completely to the locked state, a certain amount of P would exist. Added Mn^{2+} ions would then immediately form some MnP to which Ca^{2+} ions would bind to form CaMnP; this would be an immediate sink for the added Mn^{2+} ions, contrary to observation.

The above shows that all Con A molecules are in the locked conformation after equilibration with Ca^{2+} ions and contain Ca^{2+} bound in such a fashion that Mn^{2+} cannot bind without some time delay. The natural assumption is that Ca^{2+} ions are at both S1 and S2, though we have not yet done the appropriate

titration to establish this point unequivocally.[3] However, by analogy with titrations for the ternary Mn^{2+}–Con A complexes (Brown et al., 1977), and the uniform results with other ions that show S2 must be occupied for the unlocked–locked transition to occur, we assert that the locked Ca^{2+} complex formed in our experiments is CaCaPL. A single scheme, Scheme I, then subsumes all our data.

Some remarks are in order regarding the magnitude of the time constant τ_R, eq 4, which, in the absence of saccharide, is given by:

$$\tau_R = \frac{k_2 K_{MPL}}{k_{-2} k_2' K_{CPL}} \qquad (14)$$

If the binding of Mn^{2+} and Ca^{2+} ions to PL is comparable, i.e., if $K_{MPL} \simeq K_{CPL}$, and if the on-rates of Ca^{2+} to MPL and CPL are comparable, then τ_R is approximately the reciprocal of k_{-2}, the off-rate of Ca^{2+} from S2 of CaCaPL. From the results presented here, $\tau_R = 0.010$ h = 36 s, we obtain $k_{-2} \simeq 0.027$ s^{-1} at room temperature, with an activation energy of ~9 kcal M^{-1}. This time is to be compared with the several hours required to chelate metals from native Con A at (the same) pH 6.4 (Brown et al., unpublished; Harrington & Wilkins, 1978). Harrington & Wilkins (1978) have measured k_{-2} directly by observing, after addition of EDTA, the restoration of fluorescence of a quenched fluorescent saccharide bound to CaCaPL. They report $k_{-2} = 0.01$ s^{-1} at 25 °C and pH 6.4. The close agreement of our estimate with this result, obtained from experiments which monitor the kinetics of saccharide binding in contrast to ours which measure the kinetics of metal binding, gives additional confirmation of the validity of the schemes that underlie our procedures for data analysis. Moreover, comparison of the two result gives $K_{MPL}k_2/K_{CPL}k_2' = 3.6$, indicating that the kinetics of binding of Ca^{2+} and Mn^{2+} to PL, to form CaCaPL and CaMnPL respectively, are comparable.

No data exist as yet from which even an approximate value of K_{CCPL} can be made. However, if one assumes an on-rate of about 10^6 s^{-1} M^{-1} for k_2, and takes $k_{-2} = 0.01$ s^{-1}, then $K_{CCPL} = 10^{-8}$ M, an extremely small dissociation constant. Though this is a very rough estimate of the affinity of Ca^{2+} ions at S2 in CaCaPL, it may be compared with the (much greater) value $K_{MMPL} = 2 \times 10^{-5}$ M for the dissociation of Mn^{2+} from S2 in MnMnPL under similar conditions, for which the two Mn^{2+} per monomer are known to be in rapid exchange with solvent (Brown et al., 1977). Such a low dissociation constant for Ca^{2+} ions would, for the total concentrations of Ca^{2+} ions used in the present experiments, force the equilibrium in Scheme II, lower line, essentially completely to the right even if the dissociation constant of Ca^{2+} for S1 were very large, say 1 M. This is totally consistent with our observations, and the interpretation of Harrington & Wilkins (1978) based on arguments that all protein is in the CaCaPL complex before addition of Mn^{2+}.

There is an implicit assumption in the foregoing analysis that needs justification: it is assumed that the pathway for replacement of Ca^{2+} ions at S1 in CaCaPL by Mn^{2+} ions to form CaMnPL is from right to left along the upper row of Scheme III and then along the lower row from left to right. That is, it is assumed that the locked conformation is retained during the replacement processes. A possible alternate pathway involves changes of conformation: along CaCaPL → CaCaP → CaP → P, as can be traced in Scheme II, and then along the reverse set of reactions P → MnP → CaMnP → CaMnPL. We can

exclude the latter on the basis of kinetic and equilibrium considerations. Thus, we have shown that the equilibrium CaCaP ⇌ CaCaPL is essentially all to the right, and, though we have not measured the rate constant for locking, we know it to be of the order of several minutes (unpublished results). The time for unlocking would have to be very much longer. On the other hand, the derived value for τ_R, which on this alternate pathway would correspond to the unlocking time for CaCaPL, is a few minutes; the inconsistency proves the point that the alternate pathway is not relevant here. In addition, the locking time for CaMnP is highly activated and long, about 0.33 h, at 5 °C; this also makes the alternate pathway inconsistent with the observed rates of Ca^{2+} replacement by Mn^{2+}.

There is a minor effect, not yet mentioned, that has been ignored in reducing the data. It was assumed that the preference for Mn^{2+} over Ca^{2+} at S1 in the locked ternary complexes was so great that Mn^{2+} ions would displace, stoichiometrically, Ca^{2+} at S1. This is not quite so, and, though neglecting this point does not influence the derived results (within the present uncertainty), experimental conditions can be found in which the relative affinity of S1 for Mn^{2+} and Ca^{2+} ions in CaMnPL and CaCaPL, respectively, can be measured.

Interaction of Saccharide with Con A in the Presence of Metal Ions. The time dependence of binding of Mn^{2+} ions to apo-Con A equilibrated with Ca^{2+} ions, and in the presence of α-MDM, can be described by Scheme IV and eq 9–14, as seen in Figure 5. The data indicate that the rate of replacement of Ca^{2+} by Mn^{2+} is reduced by the presence of saccharide, but that the effect does not increase indefinitely as the concentration of α-MDM is increased. (Note, however, that 100 mM of α-MDGal, a weakly-binding saccharide, is required to produce the same change as 0.5 mM α-MDM, as seen in Figure 5 and Table II.) The direct inference, in terms of Scheme IV, is that Mn^{2+} ions can replace Ca^{2+} ions at S1 from SaCaCaPL. This in turn means that Ca^{2+} ions can leave SaCaCaPL without the saccharide leaving (though more slowly than from CaCaPL), and therefore that α-MDM must bind comparably, but more weakly, to PL. Thus, to first order, the saccharide-binding activity of Con A is determined by the conformation of the protein. Quantitatively, the fit in Figure 5 shows that the affintiy of α-MDM for PL is about 7% of its affinity for CaCaPL. The values for K_{SCCPL} of 0.070 and 0.24 mM at 5 and 25 °C, respectively, may be compared with the analogous results of 0.060 and 0.12 mM at 8 and 27 °C for binding of α-MDM to native Con A (Bessler et al., 1974). Thus, the binding of α-MDM to CaCaPL and to native Con A is about equally strong. This finding is similar to that of Harrington & Wilkins (1978) who showed that the affinity of 4-methylumbelliferyl α-D-mannopyranoside is essentially the same for CaCaPL, MnMnPL, and CaMnPL (native) Con A.

The affinity of α-MDM for unlocked ternary metal ion–Con A complexes is no less than 0.2 M or about 1000-fold less than K_{SCCPL}; also, the affinity of α-MDM for apo-Con A in the unlocked conformation must be less by at least another order of magnitude (Koenig et al., to be published). Thus, the change in conformation of Con A from unlocked to locked increases the binding of α-MDM by a factor 10^3, whereas going from the demetallized forms to the ternary complexes, for either conformation, increases in the saccharide affinity only about one order of magnitude. Therefore, the primary role of metals in Con A is to maintain the protein molecules in the locked conformation, which binds saccharide well. Secondarily, the presence of metals fine-tunes the structure of the saccharide-binding site so as to optimize the saccharide affinity.

The recent work of Harrington & Wilkins (1978), in which

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[3] Note Added in Proof: We have since experimentally verified that this assumption is correct.

they report studies of saccharide binding to Con A by observing either changes in fluorescence or changes in the absorption spectrum of derivatives of D-mannopyranoside, is particularly relevant to the foregoing. Their experiments are complementary to those presented here in that they observe the saccharides directly and deduce the behavior of the metal ions; we, on the other hand, observe the paramagnetic ions and deduce the binding of saccharide and nonparamagnetic metal ions. They interpret their results in terms of the two conformation states, unlocked and locked, first proposed by Brown et al. (1977) and thus their conclusions are directly comparable with ours. They find that apo-Con A with either Mn^{2+} or Ca^{2+} ions added in excess undergoes a conformation change to a state with high saccharide affinity; the time course was rapid for Ca^{2+} addition, longer for Mn^{2+} addition, and with a pH dependence much like that of the unlocked–locked transition CaMnP → CaMnPL investigated in detail by Brown et al. (1977). In the language of the schemes presented here, they produced transitions to the states MnMnPL and CaCaPL, respectively, both of which they found to have a strong affinity for their fluorescent α-MDM derivative. Indeed, they report values of 0.33 and 0.28 mM for the dissociation constants of the fluorescent saccharide to MnMnPL and CaCaPL, respectively, to be compared with 0.3 mM for the native protein (Loontiens et al., 1977).

Interaction of Saccharide with Con A in the Absence of Metal Ions. Harrington & Wilkins (1978) produced PL by removing metals from MnMnPL with EDTA, following the procedure of Brown et al. (1977), and measured saccharide binding to PL directly. They report the saccharide affinity of PL 10% to 30% of that of the locked ternary complexes, compared with our value of 7% derived using the approximations in eq 13. They also report, for the first time, a lifetime of about 3 h at 25 °C for the transition PL → P. We have performed the reverse experiment, adding sufficient α-MDM to form almost all SaPL in 11 days. This experiment not only proves that α-MDM binds mores strongly to PL than to P but, since the transition P → PL is clearly the rate-limiting step, also allows us to estimate the magnitude of the equilibrium constant K_{LP} ≡ [P]/[PL] ≲ 11 × 24/3 = 88 at 25 °C. Thus, at equilibrium, about 1% of apo-Con A is in the locked conformation, which corresponds to a free-energy difference between the locked and unlocked conformations of apo-Con A of about 3 kcal M^{-1}.

General Conclusions

A comprehensive picture begins to emerge regarding metal binding to apo-Con A. The dissociation constant of Mn^{2+} from S1 in the unlocked conformation is about $\sim10^{-2}$ mM at pH 6.4, 25 °C (Brown et al., 1977), and the dissociation of Ca^{2+} from S1 in the unlocked conformation is greater than for Mn^{2+} ions (Kalb & Levitzki, 1968). However, the dissociation constant of Ca^{2+} from S2 of the locked protein is many orders of magnitude less than for the unlocked, and much less than that of Mn^{2+} from S2. Accordingly, even with very weak binding of Ca^{2+} at S1, the strong binding of Ca^{2+} to S2 in the locked conformation would result in the ultimate formation of the ternary Ca^{2+}-Con A complex in the presence of even a small stoichiometric excess of Ca^{2+} ions. It would then appear, given the wide variety of metal ions known to bind at S1 (Shoham et al., 1973), that the primary function of divalent metal ions at S1 is to provide charge compensation of some sort that in turn allows ions to bind to S2. (Note that monovalent cations such as Na^+ and K^+, present in buffer in varying amounts, do not influence the binding of the various divalent cations, and that Gd^{3+} binds elsewhere than at S1 and S2 (Ganz et al., unpublished).) By contrast, there is a very strong preference

for Ca^{2+} ions at S2 in the locked conformation, whether S1 contains Mn^{2+}, Ca^{2+}, or by extension, any other of the many types of ions that bind there, which accounts for the presence of Ca^{2+} ions at S2 in the native protein. The apparently stronger binding of Ca^{2+} in CaMnPL compared with CaCaPL can be explained by the stronger binding of Mn^{2+} ions to S1, and a binding affinity of Ca^{2+} at S2 independent of which ion is at S1. Nonetheless, from the results above, and as found by Harrington & Wilkins (1978), strong saccharide binding requires only the presence of the locked conformation. The presence of metals at both S1 and S2, again presumably because of Coulomb forces, and most certainly so (Zimmerman & Scheraga, 1976) if our earlier identification of the conformation change with a cis–trans isomerization (Brown et al., 1977) is correct,[4] assures that the locked conformation is the one lower in energy.

The foregoing is not to suggest that saccharide binding is independent of the type of ion at S1. From previous results, it is known that binding of α-MDM, which binds about 12 Å from the ion at S1 (Brewer et al., 1973a,b; Villafranca & Viola, 1974; Alter & Magnuson, 1974), does decrease the relaxivity of Mn^{2+} ions at S1 by about 15% (Koenig et al., 1973). Thus, reciprocally, the type of metal ion at S1 must influence saccharide binding somewhat. But this is a small effect, as has been indicated.

As a final point, comment is in order regarding the recent paper by Alter et al. (1977) in which they interpret some of their data on Ca^{2+} and Mn^{2+} binding to Con A in terms of an induced cooperativity. They clearly demonstrate two significant points, that equilibration of apo-Con A with Ca^{2+} ions influences the subsequent binding of Mn^{2+} ions, and that Ca^{2+} ions bind to apo-Con A in the absence of other divalent metal ions. They are also sensitive to the various time-dependent phenomena that can occur, in that they allow a substantial time for equilibration, often as much as one week. Alter et al. (1977) interpret much of their data in terms of cooperative binding of ions at the two equivalent sites per dimer, a cooperativity that is induced by preequilibration with Ca^{2+} ions. We did not observe cooperativity of this sort previously, and have commented on this point (Brown et al., 1977); in that work, however, the fractional occupancy of S1 was maintained relatively low. In the present work, we have also maintained the occupancy of S1 low to minimize whatever complications cooperativity might produce, and thus allow a meaningful comparison of our results in the presence and absence of saccharide. We do note above that the value of τ_R increases as the fractional occupancy of S1 by Mn^{2+} in the locked protein increases, indicating that binding of Ca^{2+} at S2 is affected by the type of ion at the second S1 site of the dimer. The problem of allosteric interactions in Con A dimers requires further investigation.

Summary

We have shown that the sole addition of Ca^{2+} ions to apo-Con A induces a conformation change of the protein to a ternary Ca^{2+}-Con A complex in the locked conformation, CaCaPL. This complex binds α-MDM as tightly as does native Con A. Addition of Mn^{2+} ions to solutions of this ternary complex results in the ultimate replacement of Ca^{2+} ions at S1 by Mn^{2+} ions, to form the native protein. The kinetics of Ca^{2+} replacement are dominated by the relatively slow off-rate of Ca^{2+} ions from S2 (about 0.01 s^{-1} at 5 °C), which we investigated in some detail both in the presence and absence of

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[4] Note Added in Proof: That the conformation change is indeed a cis–trans isomerization has been confirmed recently by Reeke et al. (1978).

α-MDM. On the basis of the dependence of the kinetics of Ca^{2+} replacement on saccharide concentration, we concluded that α-MDM must also bind tightly to apo-Con A in the locked conformation, in fact, about 7% as well as to native Con A but $\sim 10^3$ times more tightly than to apo-Con A in the unlocked conformation. In addition, we demonstrated the preferential binding of α-MDM to locked apo-Con A directly: α-MDM added to P was shown to drive the equilibrium P $\rightleftarrows$ PL + Sa $\rightleftarrows$ SaPL completely to the right. That SaPL was indeed formed was demonstrated by the relaxation behavior of these solutions upon the addition of appropriate amounts of Mn^{2+} and Ca^{2+} ions, the behavior of which is unique and predictable on the basis of our earlier work (Brown et al., 1977). Finally, we have extended the multiple equilibria schemes, first introduced by Brown et al. (1977) to explain the interaction of Mn^{2+} and Ca^{2+} ions with Con A in its two conformations, to include competitive interactions for identical metal-binding sites, and to include interactions with saccharides as well.

Acknowledgments

We thank Professor Wilkins for making the Harrington and Wilkins manuscripts available to us before publication and for several mutually productive conversations that we have had. We also thank W. Ganz for communicating his data on dextran-precipitation.

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Physical Properties of *Artemia salina* Ribosomes[†]

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ABSTRACT: Eukaryotic ribosomes were isolated from the cryptobiotic embryos and from the further-developed free-swimming nauplii of the brine shrimp *Artemia salina*. Analytical boundary sedimentation and photon correlation spectroscopy yielded, respectively, the standard sedimentation and diffusion coefficients at infinite dilution, $s^{\circ}_{20,w} = 81 \pm 1$ S and $D^{\circ}_{20,w} = (1.41 \pm 0.02) \times 10^{-7}$ cm²/s, for the unfixed and formaldehyde-fixed ribosomes from different developmental stages and for ribosomes attached to a messenger RNA fragment. Also, the density increment was determined, from which the partial specific volume was derived (0.63 ± 0.01 cm³/g). Combination of the different measured parameters gives accurate values for the molecular weight (3.8 ± 0.1) × 10^6 and for size and solvation parameters. These results are compared with their counterparts for the smaller ribosomes from the prokaryote *Escherichia coli*.

The ribosome is a subcellular particle containing RNA and proteins. Engaged in protein synthesis, as a part of a polysome, it is the site of translation of messenger RNA into polypeptide sequences. It is already possible to establish a coherent picture of the physical properties of the ribosomes isolated from the prokaryote *Escherichia coli*, but the information about the larger ribosomes from eukaryotes is still much more limited (Van Holde and Hill, 1974). The difficulties in physical-chemical studies of ribosomes are caused by contamination of the solutions by other subcellular particles, by a possible conformational difference between free single ribosomes and those attached to mRNA in polysomes (Vournakis and Rich, 1971), by the pressure-dependent equilibrium between the ribosomes