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Conformational Equilibrium of Demetalized Concanavalin A†

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ABSTRACT: Concanavalin A (Con A) is known to exist in two conformations [Brown, R. D., III, Brewer, C. F., & Koenig, S. H. (1977) *Biochemistry 16*, 3883-3896] that differ in their metal ion and saccharide binding properties. The conformation that binds metal ions tightly, and which is associated with saccharide binding, has been designated as "locked" and that which binds metal ions only weakly as "unlocked". In the presence of excess metal ions, such as Mn^{2+} and Ca^{2+}, essentially 100% of the protein is in the locked conformation. The scheme proposed to explain these effects [Koenig, S. H., Brewer, C. F., & Brown, R. D., III (1978) *Biochemistry 17*, 4251-4260] predicts an equilibrium between these conformations for the apoprotein. By monitoring the solvent proton relaxation dispersion as equimolar concentrations of Mn^{2+} and Ca^{2+} are titrated, at 5 °C, into an apo-Con A solution that had been equilibrated at 25 °C, we find that 12.5% of the apoprotein is in the locked conformation, corresponding to an energy separation of 1.2 kcal mol^{-1}. We also show that these conformations can be separated by column chromatography at 5 °C and that the 100% unlocked form prepared in this way returns to the expected equilibrium mixture when kept at 25 °C.

Concanavalin A (Con A),[1] a metalloprotein isolated from the jack bean (*Canavalia ensiformis*), is a dimer below pH ~6 of molecular weight 54000 (McKenzie et al., 1972; Senear & Teller, 1981). For some time it has been known that full saccharide binding and agglutination properties of Con A require the presence of divalent cations (Yariv et al., 1968; Kalb & Levitzki, 1968; Agrawal & Goldstein, 1968; Inbar & 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 & Levitzki, 1968; Shoham et al., 1973). Recent evidence indicates that Ca^{2+} can also bind at S1 (Harrington & Wilkins, 1978; Koenig et al., 1978) and Mn^{2+} at S2 (Brown et al., 1977). S2 is not formed until S1 is occupied (Shoham et al., 1979), and occupation of both sites is believed necessary for full saccharide binding (Agrawal & Goldstein, 1968) and agglutination activity (Inbar & Sachs, 1969), though the stoichiometry of activation of Con A by Mn^{2+} has been questioned by Christie et al. (1980).

It is known that there are two conformations of Con A separated by a high energy barrier, about 22 kcal mol^{-1}, with their ground-state energies differing by only a few kilocalories per mole, the sign of this difference depending on the occupancy of S1 and S2 by metal ions (Brown et al., 1977). A major distinction between the two conformations is that the one associated with fully metalized protein (the "native" protein) has a far greater affinity for metals than the other. It was named, accordingly, the "locked" conformation, and the other, "unlocked".

It was conjectured by Brown et al. (1977) that the saccharide binding and agglutination activity of the fully metalized protein were properties associated with the locked conformation as well as with the metal ion content. The correlation of these activities with the presence of metal ions was considered to derive from the fact that metal ions lower the energy of the locked relative to the unlocked conformation, making it the predominant metalized form at equilibrium. This conjecture was subsequently verified by Harrington & Wilkins (1978) and Koenig et al. (1978). Using different methods, they showed that demetalized Con A in the higher energy, metastable, locked conformation binds saccharide quite

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[1] Abbreviations: Con A, concanavalin A with unspecified metal content; P, AP, and BAP, 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, respectively, all with the protein in the "unlocked" conformation; the suffix L (e.g., BAPL) indicates the analogous molecule in the "locked" conformation; the prefix S (e.g., SBAPL) indicates bound saccharide; M and C, Mn^{2+} and Ca^{2+} ions, respectively; EDTA, ethylenediaminetetraacetic acid; α-MDM, methyl α-D-mannopyranoside; NMRD, nuclear magnetic relaxation dispersion.

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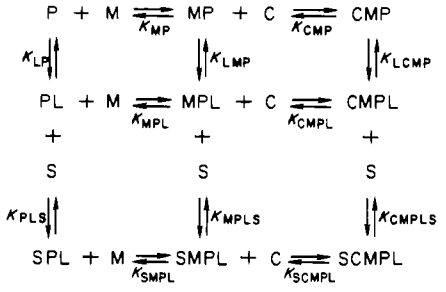
Scheme I



well. Binding by the demetalized locked conformation was found to be about 7% of that of the fully metalized locked form, a value much greater than any saccharide binding activity (monitored by using fluorescent sugars) evidenced by the unlocked conformation under comparable circumstances. The results of Brown et al. (1977) and Koenig et al. (1978) were obtained from studies of the influence of the paramagnetism of Mn^{2+}–Con A complexes in solution on the magnetic relaxation rates of solvent protons. Those of Harrington & Wilkins (1978) came from studies of the fluorescence quenching of sugars interacting with Con A. These data show that Scheme I (Brown et al., 1977; Koenig et al., 1978) describes the equilibrium and kinetic phenomena resulting from the interactions of Mn^{2+}, Ca^{2+}, and saccharide with Con A. In Scheme[2] I, P stands for apoprotein, M for Mn^{2+} at S1, C for Ca^{2+} at S2, and S for saccharide. The suffix L indicates the locked conformation, and its absence the unlocked. Species with a single metal ion have S1 occupied, and the sequence of binding of metal ions in a given species, e.g., CMPL, is read from right to left. Scheme I is a simplification in that Mn^{2+} competes with Ca^{2+} for S2 (Brown et al., 1977) and Ca^{2+} with Mn^{2+} at S1 (Harrington & Wilkins, 1978; Koenig et al., 1978). However, their respective dissociation and kinetic constants are such that, for the conditions considered below, the effect of the competition is negligible.

Scheme I has been found to have very general applicability. In addition to the studies by Wilkins and co-workers (Harrington & Wilkins, 1978; Harrington et al., 1981) using fluorescent sugars, it is supported by polarographic studies of the kinetics of binding of a variety of metal ions to Con A (Sherry et al., 1978), the UV circular dichroism studies of Cardin & Behnke (1978), and the UV difference spectroscopy results of Stroupe & Doyle (1980), as well as the extensive investigations by ^{113}Cd NMR of the binding of Cd^{2+} ions (Palmer et al., 1980).

The equilibria among the unlocked forms, top horizontal line of Scheme I, are rapid and can be regarded as instantaneous for the present study. The same is true for the kinetics of all the other horizontal processes except for the loss of metal from S2: CMPL → C + MPL and SCMPL → C + SMPL are slow. The vertical processes in Scheme I that interconvert the two conformations are slow in all cases. Since the equilibrium between CMP and CMPL is largely in favor of CMPL, the time constant for the approach to equilibrium can be taken as the first-order kinetic constant for the conversion of CMP to CMPL, or about 1.5 min at 23 °C (Grimaldi & Sykes, 1975) and 17 min at 5 °C (Brown et al., 1977), and, similarly, between P and PL, the time constant is about 3 h at room temperature and days at 5 °C (Harrington & Wilkins, 1978; Brown et al., 1977). The equilibrium ratio [PL]/[P] is unknown, though it can be inferred from the literature to be substantially in favor of P. [Brown et al. (1977) give a lower limit of [PL]/[P] of 0.01.]

It had been our experience that on titrating small concentrations of metal ions into large concentrations of apo-Con A some metal ions are initially taken up very rapidly, as though a small concentration of PL had to be saturated before P could be properly titrated [cf. Figure 4 of Brown et al. (1977)]. Also, in an experiment in which Ca^{2+} or Mn^{2+} ions were titrated into demetalized Con A and the onset of saccharide binding activity was monitored by following the quenching of saccharide fluorescence, Harrington & Wilkins (1978) observed an immediate response until about 0.3 monomer equivalent of ions was added; additional quenching would set in slowly as the remaining (unlocked) protein locked. This suggested (to us) the presence of a significant amount of PL in equilibrium solutions of apo-Con A and that the equilibrium is not so one-sided that the equilibrium value of the ratio [PL]/[P] can be considered zero when interpreting data that ostensibly measure the stoichiometry of Con A–metal–saccharide interactions.

The aim of the present study was to determine the equilibrium ratio of [PL]/[P] at 25 °C as part of a larger program to measure all the kinetic and equilibrium constants in Scheme I, generalized to include other metal ions as well. Since the time constant for the apoprotein to attain conformational equilibrium is so long at 5 °C (Brown et al., 1977), an equilibrium mixture of conformers at 25 °C can be frozen in, and analyzed, at 5 °C. Two methods were used to measure the concentration of PL in a sample of apo-Con A: (1) simultaneous titration of Mn^{2+} and Ca^{2+} into a solution of apo-Con A at 5 °C, using magnetic relaxation to measure the relative concentration of metals taken up by PL; and (2) column chromatography to separate PL from P, as an independent confirmation of the relaxation results.

The relaxation method depends on the fact that the pathway P → MP → CMP → CMPL is slow at 5 °C and rate limited by the last step, whereas the pathway PL → MPL → CMPL is rapid. Mn^{2+} aquoions and the several complexes of Mn^{2+} with Con A have identifiable contributions to the magnetic relaxation spectra of solvent protons, allowing one to monitor progress along these kinetic pathways (Brown et al., 1977). Additionally, the affinity of CMPL for its metal ions is so strong that no detectable concentration of Mn^{2+} remains in solution until essentially 100% of the S1 and S2 sites are filled, pairwise. The protocol for determining the [PL]/[P] ratio of a particular sample, then, was to titrate simultaneously equimolar Mn^{2+} and Ca^{2+} into the sample at 5 °C. The PL molecules would be occupied essentially instantaneously; this event could be monitored by the characteristic relaxation spectra of CMPL. Once all these molecules were filled, the Mn^{2+} ions form MP and, only at higher concentrations of metal ions, CMP (since the binding of Ca^{2+} ions to MP is relatively weak). The filling of P can be distinguished from the filling of PL since MP has a relaxation spectrum different from that of CMPL. (At the pH used, negligible amounts of Mn^{2+} ions are free in solution.)

In the chromatography experiments, since PL is known to bind saccharide (Harrington & Wilkins, 1978; Koenig et al., 1978), it was expected that the PL in apo-Con A equilibrated at 25 °C could be removed by passing the protein solution over a cold Sephadex column. The experiments were successful, but only because of the presence of low concentrations of adventitious metal ions in the eluting buffer that selectively bound to PL, thereby increasing its saccharide affinity, as

[2] With the exception of the dissociation constant K_{PLS}, the nomenclature is identical with that used previously (Brown et al., 1977; Koenig et al., 1978).

discussed below. It should be noted that the low concentrations (<0.06 mM) of trace metals in the buffer have little effect on the value of [PL]/[P] observed in the titration. The most they can do is reduce the total apoprotein concentration by <4% (<0.06 mM metals on 2 × 0.8 mM protein sites). Since this is an equilibrium experiment, there can be no "catalytic" effects that will change [PL]/[P].

We find a value of [PL]/[P] at equilibrium at 25 °C of 0.14 ± 0.01, corresponding to 12.5% of the apo-Con A in the locked conformation, and an energy difference of 1.2 kcal mol^{-1}. The implications of these results with regard to previous metal and saccharide binding studies are discussed.

Materials and Methods

Sample Preparation. Native Con A was obtained from Miles-Yeda (lot 172), from which apo-Con A was prepared according to the procedure described by Brown et al. (1977). Manganese chloride tetrahydrate and calcium chloride dihydrate, as well as all reagents, were obtained from Fisher Chemicals. Distilled, deionized water was used throughout. All samples were prepared and measured in pH 6.4 buffer (0.1 M potassium acetate and 0.9 M potassium chloride). Protein concentrations were determined optically at pH 5.6, using an absorbance $A^{1\%,1cm}_{280nm}$ = 12.4 (Yariv et al., 1968), and are reported as concentration of monomeric units.

Solvent Proton Relaxation Measurements. Measurements of the magnetic field dependence[3] of the spin–lattice relaxation time (T_1) of solvent protons were made by the field cycling method used previously (Brown et al., 1977; Koenig et al., 1978) and described to a limited extent by Koenig & Schillinger (1969) and Hallenga & Koenig (1976).

Chromatography. Apo-Con A (20.3 mg/mL) in pH 6.4 buffer was allowed to stand at 25 °C for 1 week, and then 2.5 mL was placed on a Sephadex G-75 column (2.3 × 27 cm) at 5 °C and eluted with the same buffer. The eluate was monitored by the ultraviolet absorbance at 280 nm, and fractions containing about 4 mL were collected. After elution with the pH 6.4 buffer, the same buffer containing 0.1 M α-MDM was used to elute the column. In a subsequent experiment, 3.0 mL of the apo-Con A solution (20.3 mg/mL) was incubated with 10 mM EDTA in the pH 6.4 buffer for an additional 4 days at 25 °C and then placed on the Sephadex G-75 column at 5 °C and eluted as described above, but with 1.0 mM EDTA in the pH 6.4 buffer.

Titration of Apo-Con A with Mn^{2+} and Ca^{2+}. Apo-Con A (0.79 mM) that had been equilibrated at 25 °C for at least 1 week was cooled to 5 °C and immediately titrated with a stock solution containing 0.0167 M $MnCl_2$ and 0.0167 M $CaCl_2$ in water. After each successive addition of microliter aliquots of the stock solution into 0.6 mL of apoprotein, the solvent proton relaxation rates of the sample were determined at 5 °C.

Similar titrations were performed on protein samples obtained from chromatography of apo-Con A on Sephadex G-75, as described above. These samples were concentrated by using an Amicon Diaflow apparatus.

Results and Analysis

Relaxation Titration. Figure 1 shows the paramagnetic contribution to the solvent proton relaxation rates, at 0.01 and 20 MHz, of a sample of 0.79 mM apo-Con A, equilibrated at 25 °C, as a function of increasing equimolar amounts of

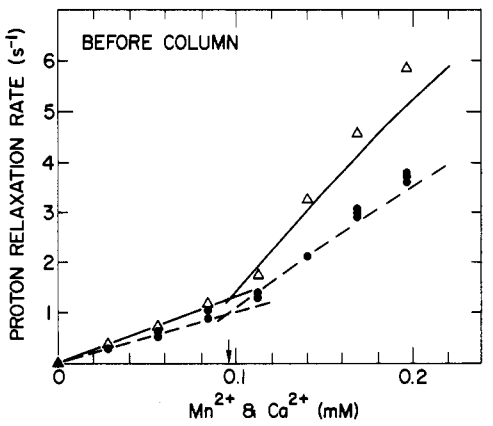


FIGURE 1: Paramagnetic contribution to the solvent proton spin–lattice relaxation rate at 0.1 (●) and 20 (△) MHz as a function of the concentration of Mn^{2+} and Ca^{2+} added to a solution of demetalized concanavalin A. The protein, 0.79 mM monomer equivalent in pH 6.4 acetate buffer (see text), was kept at 25 °C for 1 week and then brought to and kept at 5 °C for the entire experiment. The lines through the data points are the results expected from the known values of the relaxivity contributions of the several components of the system (see text), when it is assumed that 0.094 mM (indicated by the arrow) of locked, demetalized Con A (PL) was initially present in the sample.

Mn^{2+} and Ca^{2+} at 5 °C. The interpretation of the data relies on the fact that the relaxivities (relaxation rate contributions per millimolar) of CMPL at both fields are about equal and relatively low, whereas they are different and relatively high for MP (Brown et al., 1977). The circles and triangles are the data, and the curves are the theoretical results, derived as follows.

The relaxation rates to the left of the break at 0.094 mM on the horizontal scale are derived on the expectation that only CMPL is formed as the added metal ions bind to the unknown amount of PL that has been "frozen" at its 25 °C value. The relaxivity values used for CMPL were taken from Brown et al. (1977). Once all the PL sites are filled, additional metal ions initially form MP. At the higher concentrations, a small but significant amount of CMP is formed which influences the relaxivities somewhat and initiates locking by the transition CMP → CMPL. The curves to the right of the break are computed from the known relaxivities of MP (28 and 45 mM^{-1} s^{-1} at 0.01 and 20 MHz, respectively) and CMP (Brown et al., 1977), and the known value of K_{CMP} (see below). The data were taken sufficiently rapidly so that negligible locking took place. The only adjustable parameter in the fit was the concentration of metal ions, 0.094 mM, needed to position the break for a best fit to the data. Thus, 0.094 mM out of 0.79 mM apo-Con A, or about one-eighth of the total protein, takes up metals immediately to form CMPL, indicating that 12.5% of the apo-Con A was PL.

Chromatographic Results. Trace A in Figure 2 shows the elution profile obtained for a sample of apo-Con A, equilibrated at 25 °C for 1 week in pH 6.4 buffer. A large peak (I) eluted between 35 and 85 mL followed by a trailing shoulder (S) between 86 and 142 mL. In trace B, elution was continued with 0.1 M methyl α-D-mannopyranoside, and a second peak (II) appeared between 55 and 85 mL. The amount of Con A in peak I, the shoulder, and peak II was found to be 32.1, 6.1, and 10.9 mg, respectively, using an extinction coefficient for Con A in pH 6.4 buffer of $A^{1\%,1cm}_{280nm}$ = 12.8 (which was independently determined). This accounted for all the protein. The experiment was repeated with 10 mM EDTA added to the sample buffer and 1 mM added to the elution buffer (trace C). This time all of the protein was observed to elute at a position close to that of peak I.

[3] 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.

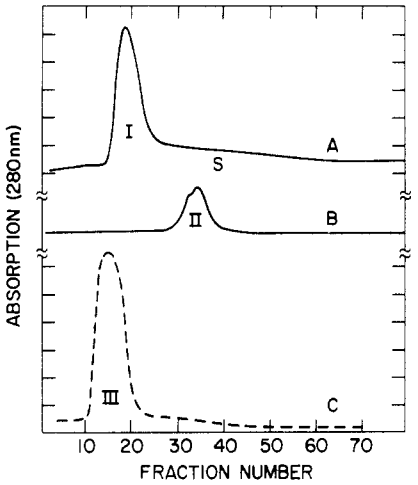


FIGURE 2: Elution profiles from a Sephadex column at 5 °C of apo-Con A that had been equilibrated for 1 week at 25 °C, under the following conditions: curve A, eluted with buffer only; curve B, second elution (after A) with buffer containing 0.1 M α-MDM; curve C, initial conditions same as those for curve A; eluted with buffer containing 1 mM EDTA.

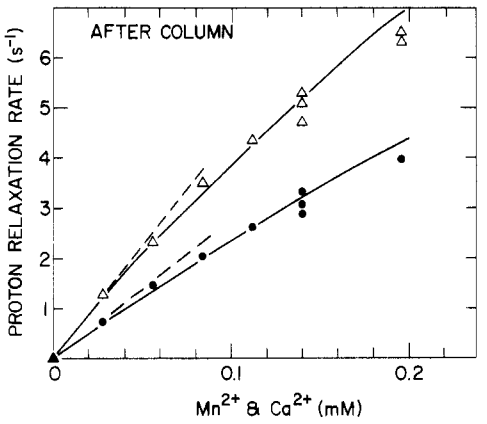


FIGURE 3: Results for an experiment similar to that of Figure 1, except that the demetalized protein was concentrated from the eluate under peak I, Figure 2. The sample was maintained at 5 °C throughout. The solid curves through the data points were computed by assuming that no locked, demetalized protein was present in the sample. No adjustable parameters were used to deduce the curves other than a refinement of the value of K_{CMP} (the dissociation constant of CMP, the Ca^{2+}–Mn^{2+}–Con A unlocked ternary complex) to obtain a best fit to the data. The dashed lines indicate the data expected when the dissociation constant is assumed infinite.

Relaxation Titration of Chromatographed Protein. The data in Figure 3 are from an experiment identical in all respects with that of Figure 1 except that the apo-Con A used was from peak I, Figure 2, maintained at 5 °C. The break observed in Figure 1 is clearly absent here; only the high-relaxivity form appears. The PL fraction has been removed by chromatography. The curved lines through the data are derived by using the same relaxivities as for Figure 1, assuming that mainly MP and a small amount of CMP are formed in known concentrations (since the respective dissociation constants are known). Since there is a rather large uncertainty in the published value of K_{CMP} [cf. Figure 9 of Brown et al. (1977)], the value 0.3 mM, which gives the best fit to these data, was used. The dashed lines show the relaxation rates expected if only MP were formed; the curvature and resulting deviation from these lines yield the value used for K_{CMP}. At the higher concentrations of metal ions, locking and therefore a slow, time-dependent downward drift of the data are observed, as indicated by a spread in the values of successive points at the same concentration of metal ions, in the right-hand half of the figure. These multiple data points give a qualitative measure of the scale of these unavoidable effects; this slight drift in no way influences the conclusions drawn below. Except for the optimization of the value of K_{CMP}, the curves through the data points (Figure 3) involve no adjustable parameters. The excellent fit, however, depends on the validity of Scheme I in detail. The parameters for the curves in Figure 3 were used without alteration to fit the data of Figure 1, and to fit the data in Figure 4 below.

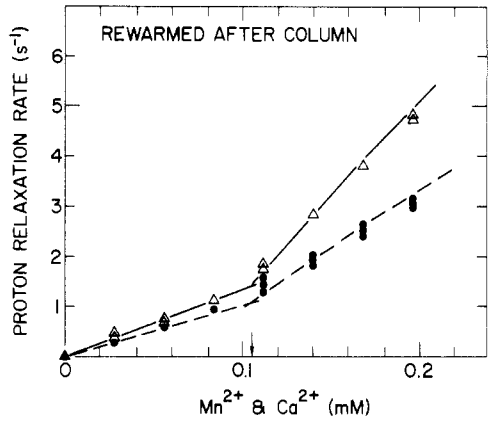


FIGURE 4: Repeat of the experiment of Figure 3 with an aliquot of the same protein that, however, was brought to 25 °C and maintained there for 1 week, and then recooled to 5 °C. The results, which should repeat those of Figure 1, have a break at 0.106 mM, indicated by the arrow.

Figure 4 shows results for an aliquot of the peak I material used in Figure 3, after it was equilibrated at 25 °C for 4 days before recooling to 5 °C. The presumption is that the 25 °C equilibrium [PL]/[P] ratio should have been restored, and therefore the data should repeat those of Figure 1. That such was the case is clear; the break has returned, at 0.106 mM, as obtained from the analysis procedures used for Figure 1. The data of Figures 1 and 4 are so similar that there can be no doubt that warming a sample of P restores the equilibrium ratio of [PL] and [P].

The results of Figures 1 and 4, when averaged, give $1/K_{LP} \equiv [PL]/[P] = 0.14 \pm 0.01$ at 25 °C, pH 6.4, corresponding to a difference in energy for the two conformations of 1.2 kcal mol^{-1}.

Discussion

The main result, that 12.5% of apo-Con A is PL at equilibrium at 25 °C, has implications for the qualitative aspects of a variety of experiments reported in the literature dealing with the metal ion and saccharide binding properties of Con A. For example, upon addition of Mn^{2+} ions to a sample of apo-Con A containing indicator amounts of fluorescent saccharide, both Harrington & Wilkins (1978) and Christie et al. (1979) observed prompt quenching of the fluorescence followed by a slow increase of quenching with time. Harrington and Wilkins say that the "results would indicate that the unlocked (metalized) form can bind (saccharide) to a limited extent". Christie et al. (1979) do not remark on this phenomena, though the effect corresponds to quenching of ~10% of the total fluorescence (cf. their Figure 4a). The present findings predict an immediate 12.5% quenching due to uptake of Mn^{2+} to form the MMPL adduct [cf. Brown et al. (1977)], which is known to bind saccharide as well as does CMPL (Harrington & Wilkins, 1978). This prompt process should be followed by a slow increase in quenching as MMP converts to form MMPL, as is observed. By contrast, this qualitative feature of the data would be absent if the PL

content of apo-Con A at equilibrium were negligibly small.

The above examples point out the need to be aware of the significant amount of PL present in equilibrium solutions of apo-Con A during studies designed to investigate the relationship between metal ion binding and saccharide binding activity of the protein. In particular, experiments in which small amounts of fluorescent saccharide are used to monitor the activation of a much larger amount of apo-Con A by added metal ions can lead to a confusing kinetic and thermodynamic picture of ligand binding to the protein.

The chromatographic results, which reinforce the interpretation of the data (Figure 1), are significant in their own right. Chromatography on a Sephadex G-75 column at 5 °C of apo-Con A brought to conformational equilibrium at 25 °C gives markedly different results in the presence and absence of EDTA in the eluting buffer. Only one peak (III, Figure 2), containing all the protein, elutes from the column in the presence of EDTA whereas three fractions (I, S, and II) come off in its absence. From the titration results discussed above, it is evident that peak I corresponds to P. That P does not separate from PL on the column in the presence of EDTA strongly suggests that PL, though it binds saccharide, is not retained on the column. It is well-known that CMPL (or Con A with any of a number of divalent metal ions at S1 and Ca^{2+} at S2) will bind to Sephadex G-75 and can be eluted by a solution containing an appropriate saccharide, such as α-MDM. Thus, peak II is most likely a metalized form of PL; the large volume of buffer necessary to elute the protein in the absence of EDTA provides sufficient amounts of adventitious metal ions to produce metalized "active" Con A only from PL.

The result that 22% of the protein is in peak II and 12% is in the shoulder (which disappears in the presence of EDTA) is consistent with the 12.5% PL being randomly distributed among an equilibrium mixture of dimers and tetramers when we assume that dimers of PL and P, and tetramers with at least two PL monomers, will bind strongly to Sephadex (peak II), while those tetramers with only a single PL are more weakly bound and form the shoulder. Since a random distribution of the 12.5% PL over a completely tetrameric protein would predict 33% of the protein in the shoulder, if we assume our sample to be 70% dimers (McKenzie et al., 1972; Senear & Teller, 1981), then approximately 10% of the protein would be expected in the shoulder and 19% in peak II, which agree quite well with the observed values of 12 and 22%, respectively.

It is now established that an equilibrium mixture of demetalized Con A is predominately (87%) in the unlocked conformation, and it is known that the fully metalized equilibrium is essentially totally in the locked conformation at 25 °C. (It should be noted that the value of K_{LMP} is as yet unknown.)

Finally, a remark is in order regarding monomer–monomer interactions. A comparison of the relaxation and chromatographic data indicates that the distribution of PL monomers among the (at least) dimeric apo-Con A molecules is essentially random. Though we have reported previously (Koenig et al., 1978) that the dissociation rate for the reaction CCPL → C + CPL may vary by a factor of 2 according to the metal ion occupancy of the other monomer of the dimer molecule, the results of the present study give no evidence of cooperativity in the case of PL–monomer interactions.

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