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KINETICS OF CONFORMATIONAL TRANSITIONS OF DEMETALIZED CONCANAVALIN A

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Demetalized Concanavalin A exists in two conformational states, known as locked (PL) and unlocked (P) [Brown et al., Biochemistry 16, 3883 (1977)]. The equilibrium ratio [PL]/[P] is 0.14±0.01 at 25 °C, pH 6.4 [Brown et al., Biochemistry 21, 465 (1982)]. We now report values of the rate constants for the $P \rightleftarrows PL$ equilibrium; $k_1 = (33 \pm 4\ h)^{-1}$ and $k_{-1} = (4.6 \pm 0.6\ h)^{-1}$ for the P → PL and PL → P transitions, respectively, at 25 °C, pH 6.4. The experiments utilize the fact that saccharide binds to PL [Koenig et al., Biochemistry 17, 4251 (1978)], producing a time-dependent increase in the total concentration of locked forms at equilibrium, and use a new technique for measuring this concentration.

Brown et al. (1) discovered that there exist two conformations of Con A[1] which differ in their affinity for metal ions and saccharides. The conformational equilibrium is predominantly in favor of one conformer (called "locked") when two metal ions are bound per protein monomer; the equilibrium is in favor of the "unlocked" conformation when the protein is demetalized. Scheme I describes the multiple equilibria that occur among apo-Con A and its binary and ternary complexes with metal ions as originally proposed by Brown et al. (1), and as corroborated subsequently by ourselves (2,3) and others (4,5,7,8).

The two sets of horizontal equilibria in Scheme I indicate the sequential binding of metal ions: A to site S1 (the "transition metal site"), after which metal ion B may bind to an adjacent site S2 (the "Ca^{2+} site"). P represents demetalized protein. The upper line represents the equilibria of the metal ions with the unlocked conformation; and the lower line

[1] Abbreviations used: Con A, concanavalin A with unspecified metal ion content and conformational state; P, AP, and BAP, respectively apo-Con A, Con A with metal ion A at site S1, and Con A with metal ion B at site S2 and A at S1, all in the conformation known as "unlocked". The suffix L indicates the same complexes in the "locked" conformation. C and M are used specifically to represent Ca^{2+} and Mn^{2+} in these complexes as in CMPL; α-MDM, methyl-α-D-mannopyranoside; EDTA, ethylenediaminetetraacetic acid.

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$$\begin{array}{ccccc} & K_{AP} & & K_{BAP} & \\ A + P & \rightleftarrows & AP + B & \rightleftarrows & BAP \\ K_{LP} \uparrow\downarrow & & \uparrow\downarrow K_{LAP} & & \uparrow\downarrow K_{LBAP} \\ A + PL & \rightleftarrows & APL + B & \rightleftarrows & BAPL \\ & K_{APL} & & K_{BAPL} & \end{array}$$

Scheme I

the analogous equilibria with the locked conformation, indicated by the suffix L. The affinity of the locked conformation for metal ions, and the locked ternary complexes for certain saccharides, is much greater than that of the unlocked conformation. The two conformations have comparable free energies, with relative values that depend on the particular complex, but are separated by a large activation barrier (~20 Kcal M^{-1}) that makes the conformational interconversion, and therefore the establishment of thermal equilibrium, relatively slow (minutes to many hours).

Our continuing interest has been to measure the equilibrium constants and the related rate constants for the equilibria of Scheme I, and the saccharide binding constants of the many complexes. The question exists as to the relative extent to which conformation and binding of metals contribute to the specific saccharide affinity of various ternary metal-Con A complexes, including MMPL, CMPL, and CCPL (where M indicates Mn^{2+} and C, Ca^{2+}).

Brown et al. (3) recently reported that the equilibrium ratio $[PL]/[P] \equiv 1/K_{LP} = 0.14 \pm .01$ at 25 °C, pH 6.4. Though there was an initial questioning of this result (9), there is now general agreement that a small, but readily detectable, fraction of apo-Con A is in the locked conformation at equilibrium (10). The related rate processes are unknown, though one can infer, using Scheme I, that the remark of Harrington and Wilkins (8) "the protein remaining after treatment of [Mn-Con A] with EDTA [to form PL] lost its sugar binding with a half-life of about 2 h at pH 5.0 and faster at higher pH's" relates directly to the rate constant k_{-1} for the PL→P transition. From the known value of K_{LP}, one can estimate $\lesssim$ 20 h for the reciprocal of the rate constant k_1 for the P→PL transition. We have now determined the two rate constants directly, and find $1/k_1 = 33 \pm 4$ h and $1/k_{-1} = 4.6 \pm 0.6$ h at 25 °C, pH 6.4.

MATERIALS AND METHODS

Sample Preparation. Native Con A was obtained from Miles-Yeda (Lot 55L) from which apo-Con A was prepared (1). Distilled, deionized water was used throughout. All samples, 0.6 ml, were in pH 6.4 buffer (0.1 M potassium acetate, 0.9 M potassium chloride). Buffer components and manganese chloride tetrahydrate were obtained from Fisher Chemical, and α-MDM from Pfanstiehl. Protein concentration was determined spectrophotometrically at the end of each experiment by dilution into pH 5.6 buffer, using an absorbance $A_{280nm}^{1\%,1cm} = 12.4$, and is reported in monomeric units. Saccharide was added as the solid, and Mn^{2+} was added in μl amounts from a 0.06 M stock solution.

Proton Relaxation Measurements. Measurements of the magnetic field dependence of the spin-lattice relaxation time (T_1) of solvent protons were made by the field cycling method used previously (1,2,3,6,11).

Methodology. The experimental method depends explicitly on two facts. The first is that saccharide binds measurably to PL and negligibly to P; the dissociation constant for the complex of PL with α-MDM, K_{SPL}, is 29 mM (11). Therefore, apo-Con A has its equilibrium shifted toward the locked conformation upon addition of α-MDM by the reaction

$$P \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} PL + S \overset{K_{SPL}}{\rightleftarrows} SPL \quad . \tag{1}$$

We demonstrate this shift directly, and measure the time course for attaining the new equilibrium, by measuring [PL] + [SPL], the total concentration of locked conformers, as a function of time after a fixed concentration of α-MDM has been introduced into apo-Con A. This becomes possible because of a second fact, recently discovered and reported elsewhere (6,11): binding of α-MDM to the ternary complex of MMPL is much stronger than to either MPL or PL (Scheme I, where both A and B represent Mn^{2+}) so that Mn^{2+} ions go onto PL essentially pairwise in the presence of, for example, 100 mM α-MDM, producing the quaternary saccharide-metal-Con A complex quantitatively, until all locked protein is saturated. Excess Mn^{2+} either binds to the remaining unlocked protein or remains free in solution. These several forms of Mn^{2+} ion can be readily distinguished using our magnetic relaxation techniques (1-3).

RESULTS AND ANALYSIS

Figure 1 summarizes results for one equilibrium and two quasi-equilibrium sets of conditions. The data points extending furthest to the right show the relaxation rates of solvent protons at three fields (given in units of their respective proton Larmor frequencies) for 11 aliquots of a sample of 0.39 mM apo-Con A to which 100 mM α-MDM and various concentration of Mn^{2+} ions were added. The samples were then equilibrated at 25 °C and measured at 5 °C (cf. Figure 5 of Brewer et al. (6)). The break in the data near 0.8 mM Mn^{2+} ions per 0.39 mM Con A monomer is clear; the dashed curves to the right of the break are the results anticipated if all subsequent Mn^{2+} ions remain aquoions. These data

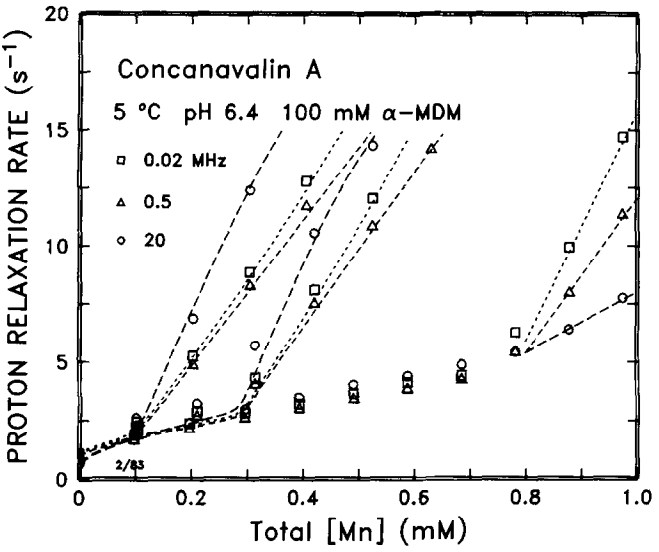

Figure 1. Spin-lattice relaxation rates of solvent protons in solutions of Con-A, as a function of total added Mn^{2+} ions, at three values of magnetic field corresponding to Larmor frequencies of 0.02, 0.5, and 20 MHz. The samples were all within ±2% of 0.38 mM apo-Con A (in monomer units), in pH 6.4 buffer (0.1 M potassium acetate and 0.9 M potassium chloride). All samples contained 100 mM α-MDM. Data were taken at 5 °C. The data with a break near 0.8 mM total Mn^{2+} are for samples that were maintained at room temperature for over two weeks to allow conformational equilibrium to be reached. The dashed curves through the data to the right of the break represent the expected results assuming that all added Mn^{2+} become aquoions once 2:1 Mn^{2+} to Con A stoichiometry is reached. The set of data with a break near 0.1 mM total Mn^{2+} result from a titration of Mn^{2+} into a sample that was allowed to reach conformational equilibrium at 25 °C in the absence of saccharide, the sample then cooled to 5 °C, α-MDM added, and the titration with Mn^{2+} performed. The dashed curves through these data points are the anticipated results, obtained using the known values of the equilibrium constant, K_{LP}, the dissociation constants that enter Scheme I, and the relaxation contributions of the several species present (cf. reference 6). No adjustable parameters are involved. The center set of data results from an experiment that differs from the foregoing only in that α-MDM was added at 25 °C, and the sample maintained warm for four days, after which it was cooled to 5 °C and the titration performed. The curves through the data points are obtained as before except that a shift in the conformational equilibrium of apo-Con A in the presence of 100 mM α-MDM, as expressed by eq. 1, was considered. The curves were calculated for a value of 29 mM for the dissociation constant K_{SPL} of the α-MDM locked apo-Con A complex at 25 °C, pH 6.4.

indicate the pairwise binding of Mn^{2+} to PL in the presence of α-MDM to form SMMPL, a low relaxivity species (6).

The left-most set of data points, those that break near 0.1 mM total Mn^{2+}, are for a sample of apo-Con A, equilibrated at 25 °C, which was then cooled to 5 °C to maintain the 25 °C conformational equilibrium. 100 mM α-MDM was then added, and the titration performed immediately. The break, due to the PL initially present in the sample, is a third measure of K_{LP} (3,10), and identical to the earlier results ($1/K_{LP}$ = 0.14). The dashed lines

are the results expected, based on the known values of all pertinent parameters (6). No adjustable parameters enter the computation of these curves.

The middle set of data and curves, with a break near 0.3 mM total Mn^{2+}, result from an experiment identical to the one above, except that the apo-Con A sample was allowed to equilibrate with 100 mM α-MDM at 25 °C (4 d). The sample was then cooled to 5 °C and titrated with Mn^{2+}. The curves through the data are those expected (6) using one new parameter, the dissociation constant of the α-MDM-PL complex, K_{SPL} = 29 mM, a value obtained from this and more extensive results (11).

The two left-most sets of data, Figure 1, may be regarded as the beginning and end points of a kinetic run in which the kinetics of the approach to a new equilibrium (eq. 1) are followed. The kinetics of the P⇄PL equilibrium is, of course, the rate limiting process by many orders of magnitude. We have repeated the foregoing experiments, taking aliquots from a stock solution kept at 25 °C at various times after the initial addition of 100 mM α-MDM. All aliquots were cooled to 5 °C, titrated with Mn^{2+}, and the data analyzed to obtain the position of the break in the titration data. The results are shown in Figure 2,

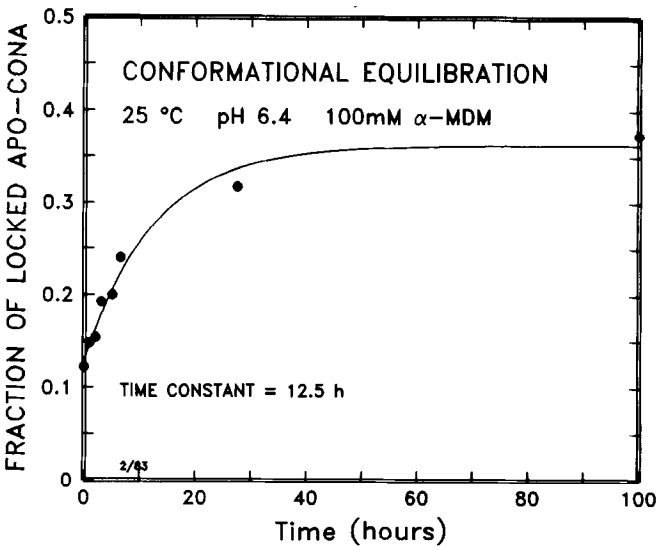

Figure 2. Experiments similar to the central one of Figure 1 were performed with the samples maintained at 25 °C for a variable time after the α-MDM was added. The samples were then cooled, the titrations performed, and the break in the data noted. These results, expressed as the fraction of apo-Con A in the locked conformation, are plotted here as a function of time. The curve through the data points result from a least-squares analysis of the data, assuming an exponential growth of the fraction of locked protein from its initial value. A time constant of 12.5±1 h was found.

expressed as the fraction of protein in the locked conformation found at a given time after addition of α-MDM. The curve through the data points results from a least-squares comparison of the data with a simple exponential growth curve, using three unknown parameters: the initial point, the end point, and the time constant. We find $\tau_S = 12.5 \pm 1$ h.

The value of τ_S is a function of [S] (the concentration of α-MDM). Referring to eq. 1, in the limit of S→∞, all PL will be converted to SPL; there will be no contribution of the back reaction PL→P to τ_S, which should then approach $1/k_1$. In the other limit, S→0, the back reaction will dominate the kinetics. It is not difficult to show that the correct expression for τ_S as a function of [S] is

$$\tau_S = \tau_o\left(\frac{1 + ([S]/K_{SPL})}{1 + ([S]\tau_o k_1/K_{SPL})}\right) \tag{2}$$

where

$$1/\tau_o = k_1 + k_{-1} = k_{-1}(1 + K_{LP}) \tag{3}$$

$$K_{LP} = k_1/k_{-1} \ . \tag{4}$$

Using eqs. 2 and 3, $\tau_S = 12.5$ h from Figure 2, $K_{SPL} = 29$ mM (6) and $1/K_{LP} = 0.14$ from previous work (3,10), we compute that $1/k_1 = 33 \pm 4$ h and $1/k_{-1} = 4.6 \pm 0.6$ h. The uncertainty includes that of K_{LP}(3).

DISCUSSION

The present experiments utilize the fact that when α-MDM binds to PL there is a shift in the distribution of conformers found in the absence of saccharide towards an increased concentration of locked forms, and use a new technique for measuring this concentration. The magnitude of the binding of α-MDM to PL was not previously known, though the existence of the α-MDM-PL complex had been demonstrated by Koenig et al. (2), and can be inferred from the results of Harrington and Wilkins (8) when the latter are interpreted according to Scheme I. On the basis of more extensive equilibrium data (11) than just the end point of Figure 2, we find a value of 29 mM for K_{SPL}, the dissociation constant of the α-MDM-PL complex. Here we establish the method, utilizing mass action effects as indicated in eq. 1, and use it to study the approach to the new equilibrium condi-

tions produced when α-MDM is added to apo-Con A. Using this procedure, we have obtained values for the rate constants for the P⇄PL experiment at 25 °C, pH 6.4; $1/k_1$ and $1/k_{-1}$ are 33±4 h and 4.6±0.6 h for the P→PL and PL→P transitions, respectively. The latter value is somewhat longer than the approximate value (<3 h) that may be inferred from Harrington and Wilkins (8). The present results represent the first direct kinetic measurements of the rates of the P⇄PL transitions of apo-Con A.

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