Kinetic Parameters for the Binding of p-Nitrophenyl α-D-Mannopyranoside to Concanavalin A[1]

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Received April 7, 1975

Binding of the chromogenic ligand p-nitrophenyl α-D-mannopyranoside to concanavalin A was studied in a stopped-flow spectrometer. Formation of the protein–ligand complex could be represented as a simple one-step process. No kinetic evidence could be obtained for a ligand-induced change in the conformation of concanavalin A, although the existence of such a conformational change was not excluded. The entire change in absorbance produced on ligand binding occurred in the monophasic process monitored in the stopped-flow spectrometer. The value of the apparent second-order rate constant (k_a) for complex formation $(k_a = 54,000 \text{ s}^{-1} \text{ M}^{-1}$ at 25°C, pH 5.0, Γ/2 0.5) was independent of the protein concentration when the protein was in the range of 233–831 μM in combining sites and in excess of the ligand. The apparent first-order rate constant (k_{-a}) for dissociation of the complex was obtained from the rate constant for the decomposition of the complex upon the addition of excess methyl α-D-mannopyranoside $(k_{-a} = 6.2 \text{ s}^{-1}$ at 25°C, pH 5.0, Γ/2 0.5). The ratio k_a/k_{-a} $(0.9 \times 10^4 \text{ M}^{-1})$ was in reasonable agreement with value of $1.1 \pm 0.1 \times 10^4 \text{ M}^{-1}$ determined for the equilibrium constant for complex formation by ultraviolet difference spectrometry. Plots of $\ln(k_a/T)$ and $\ln(k_{-a}/T)$ vs $1/T$ were linear (T is temperature) and were used to evaluate activation parameters. The enthalpies of activation for formation and dissociation of the complex are 9.5 ± 0.3 and 16.8 ± 0.2 kcal/mol, respectively. The unitary entropies of activation for formation and dissociation of the complex are 2.8 ± 1.1 and 1.3 ± 0.7 entropy units, respectively. These entropy changes are much less than those usually associated with substantial changes in the conformation of proteins.

Evidence has been presented which appears to support the hypothesis that carbohydrate ligands induce a conformational change in the lectin conconavalin A (1–7). Ligands induce changes in circular dichroic spectra (1–2) and absorption spectra (3) of the protein. The fact that these perturbations in spectra of concanavalin A occur in the 250–300-nm region suggests that ligand binding alters the environment of amino acids with aromatic side chains. Doyle *et al.* (4) have provided further evidence for a ligand-induced alteration in the environment of tryptophanyl

residues in concanavalin A. These workers found that modification of some tryptophanyl residues in concanavalin A with 2-(bromoethyl)-4-nitrophenol resulted in a protein for which ligand binding was accompanied by a change in the absorption spectrum of the nitrophenol reporter group. The observations that the presence of ligand in solutions of concanavalin A results in an increased resistance of the protein against denaturation on heating and against proteolysis on digestion with pronase have also been presented as evidence for a ligand-induced change in the conformation of concanavalin A (4). The observation that binding of carbohydrate ligands slightly alters the spin lattice relaxation time of the protons of water molecules bound to the Mn^{2+} of concanavalin A also suggests that carbohydrate binding to concanavalin A induces a conformational

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change which causes a small change in the environment at the Mn^{2+} binding site of concanavalin A (5–7). These observations coupled with the observations of second-order rate constants for binding of ligands to concanavalin A (10^4–10^5 s^{-1} M^{-1}) which are much slower than those for a diffusion-controlled process are consistent with the proposal of Brewer et al. (8) that the pathway for ligand binding to concanavalin A is given by Eq [1].

$$P + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PD_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PD_x. \quad [1]$$
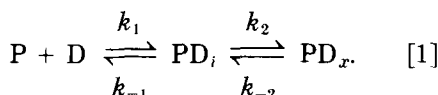
In this process, concanavalin 'A is envisaged as undergoing a slow change in conformation after the initial protein–ligand complex, PD_i, is formed. Although the conformational transition from PD_i to PD_x has not been observed directly, Brewer et al. (8) have presented as evidence for its existence, the low activation energy (2.5–5 kcal/mol) associated with the observed second-order rate constant (k_a) for binding of ^{13}C-enriched α- and β-isomers of methyl D-glucopyranoside to concanavalin A, as determined from measurements of relaxation times for ^{13}C carbon magnetic resonance. Brewer et al. (8) pointed out that if the concentration of PD_i is always low, $k_a = k_1k_2/k_{-1}$, so that the small temperature coefficient of k_a could be explained by assuming $K_1 = (k_1/k_{-1})$ decreases with increasing temperature almost as fast as k_2 increases with increasing temperature. However, the possibility cannot be rigorously excluded that binding is a simple one-step process with a low activation energy.

In this work, the process by which concanavalin A binds ligands is further characterized from a study in which a stopped-flow spectrometer is used to follow directly the binding of p-nitrophenyl α-D-mannopyranoside to concanavalin A and to determine the temperature dependence of the rate constants associated with the binding process.

MATERIALS AND METHODS

Concanavalin A obtained as a lyophilized powder, 16.7% by weight sodium chloride and p-nitro-

phenyl α-D-mannopyranoside were purchased from Calbiochem. Methyl α-D-mannopyranoside was obtained from Pfanstiehl Laboratories. Solutions were prepared using water obtained by passing the distilled water supplied to the laboratory through a Barnstead demineralizer and redistilling it in an all-glass still. All other chemicals used were Mallinckrodt, Baker–Adamson or Fisher analytical reagents.

Kinetic and equilibrium studies were carried out in acetate buffer at pH 5.0, containing 0.1 mM $MnCl_2$ and 0.1 mM $CaCl_2$ and adjusted to $\Gamma/2$ 0.5 with NaCl. The total concentration of acetate plus acetic acid was 0.5 M and the pH of the solution changed by less than 0.05 unit over the temperature range used in this study (9–37°C). Ultraviolet difference spectra were determined using a Cary spectrophotometer, Model 118, equipped with jacketted cuvette compartments for temperature control (25.0 ± 0.2°C) by circulating water. Reaction rates were followed using an Aminco–Morrow stopped-flow apparatus attached to a Beckman monochromator equipped with a logarithmic photometer. An IT 1 flexible, Teflon-sheathed thermocouple probe was inserted into the exit port of the stopped-flow observation cell to monitor the temperature of the reacting solution. The temperature of the reacting solution was monitored to ±0.1°C using this probe and a BAT 8 digital thermometer from the Bailey Instruments Co., Saddle Brook, N. J. The probe and digital thermometer were calibrated by using an NBS thermometer prior to installation in the stopped-flow apparatus. The calibration correction was 0.2°C or less. The temperature of the reacting solutions was controlled by circulating water from a thermostated bath through the jackets surrounding the drive syringes and the observation cell.

The concentration of p-nitrophenyl α-D-mannopyranoside was determined spectrophotometrically from the absorbance of solutions of this compound at 305 nm by using a molar absorptivity of 10,000 cm^{-1} M^{-1} (3). The concanavalin A concentrations specified in this work refer to the concentration of combining sites. The concentration of concanavalin A combining sites was determined from the absorbance of solutions of concanavalin A at 280 nm using a molar absorptivity of 28,770 cm^{-1} M^{-1}. This value was determined by preparing a stock solution of concanavalin A such that the uv difference spectrum generated when this solution was mixed with an equal volume of 100 μM p-nitrophenyl α-D-mannopyranoside was the same as the uv difference spectrum generated when a twofold dilution of the concanavalin A stock solution was mixed with an equal volume of 200 μM p-nitrophenyl α-D-mannopyranoside. The stock solution of concanavalin A must be 200 μM in combining sites and the molar absorptivity for concanavalin A combining sites was calculated from the absorbance at 280 nm of a diluted aliquot of the stock solution.

This molar absorptivity is essentially identical to the value of 29,070 cm^{-1} M^{-1} calculated using the value $E_{1\ cm}^{1\ \%}$ = 11.4 (9) and a molecular weight of 25,500 for the subunit (10).

The rate constant, k_{obs}, for the first-order approach of absorbance to its final value when concanavalin A was mixed with p-nitrophenyl α-D-mannopyranoside was evaluated from the slopes of linear plots of ln $|A_t - A_f|$ vs time. The absorbance at time t and the final absorbance are represented by A_t and A_f, respectively. In these experiments, the concentrations after mixing were 233–831 μM concanavalin A combining sites and 20–40 μM p-nitrophenyl α-D-mannopyranoside. The slope of similar plots of ln $|A_t - A_f|$ vs time gave the rate constant, k_{-a}, for the first-order approach of the absorbance to its final value when a solution containing 80–800 μM concanavalin A combining sites and 40–200 μm p-nitrophenyl α-D-mannopyranoside was mixed with an equal volume of 40–200 mM methyl α-D-mannopyranoside. The total change in absorbance associated with the stopped-flow trace was usually above 0.03 absorbance unit at 317 nm. Rate constants were determined from measurements at 317 nm. Changing the wavelength to 325 nm or halving the spectral bandwidth of the incident beam (usually 2 nm) had no effect on measured rate constants. All rate constants reported in this work are the average of at least two values that were within 10% of each other. Equation [2] was used to evaluate the parameter k_a.

$$k_a = (k_{obs} - k_{-a})/[P], \qquad [2]$$

where k_{obs} was measured at a concentration of concanavalin A combining sites equal to [P] at temperature T, and k_{-a} was obtained from a plot of a graph of ln (k_{-a}/T) vs $1/T$. The experimentally determined parameters k_{obs}, k_a and k_{-a} can be related to the individual rate constants of a given pathway for ligand binding. For the case when ligand binding proceeds via the one-step reaction,[3]

$$P + D \underset{k'_{-1}}{\overset{k_1'}{\rightleftharpoons}} PD; \qquad [3]$$
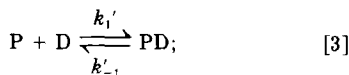
when [P] $\gg$ [D]

$$k_{obs} = k'_{-1} + k_1' [P]. \qquad [4]$$

If $k_{-a} = k'_{-1}$, $k_a = k_1'$ (compare Eq. [2] and [4]), the equality $k_{-a} = k'_{-1}$ can be tested by comparing the measured value of k_{-a} with the intercept of a plot of k_{obs} vs [P].

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[3] Equation [4] relates the observed rate constant for the approach to equilibrium by a first-order process to the sum of the pseudo-first-order rate constant (k_1' [P]) for the forward reaction and the first-order rate constant (k'_{-1}) for the reverse reaction. A derivation of this relationship for a first-order reversible reaction is presented in Ref. (11).

For the case where ligand binding proceeds via the two-step process of Eq. [1]; when [P] $\gg$ [D] and the second step is rate controlling ($k_{-1} \gg k_2 + k_{-2}$),[4]

$$k_{obs} = k_{-2} + k_2K_1[P]/(1 + K_1[P]) \qquad [5]$$

Comparing Eq. [2] and [5] it is seen that if $k_{-a} = k_{-2}$,

$$k_a = k_2K_1/(1 + K_1 [P]). \qquad [6]$$

Activation parameters were determined by interpreting linear plots of ln (k_r/T) vs $1/T$ according to the relationship

$$\ln \frac{k_r}{T} = \frac{-\Delta H\ddagger}{R} \frac{1}{T} + \frac{\Delta S\ddagger}{R} + \ln \frac{\bar{k}}{h}, \qquad [7]$$

where k_r is the rate constant (k_a or k_{-a}) and $\bar{k}/h$ is the ratio of the Boltzmann constant to the Planck constant. The entropy of activation for association contains the entropy change for bringing two molecules together in a 1 M solution. Unitary entropies of activation which do not contain this entropy of mixing were estimated using the relationship (12)

$$\Delta S_u\ddagger = \Delta S\ddagger + 7.98. \qquad [8]$$

For dissociation, there is no change in the number of molecules in the formation of the activated complex and $\Delta S_u\ddagger = \Delta S\ddagger$.

RESULTS AND DISCUSSION

The binding of p-nitrophenyl α-D-mannopyranoside to concanavalin A results in a change in the molar absorptivity of this chromogenic ligand (3). Gray and Glew (13) have shown that binding of this ligand to concanavalin A can be followed in a stopped-flow spectrometer. In theory, it should be possible to evaluate the individual rate and equilibrium constants of Eq. [1] or at least set limits on their relative magnitudes from studies of the kinetics of binding of a chromogenic ligand to concanavalin A. The dependence of the first-order rate constant (k_{obs}) for the approach of the absorbance to its final value, when concanavalin A is mixed with chromogenic ligand is given by Eq. 5, provided [P] $\gg$ [D] and the transition PD$_i$ $\rightleftharpoons$ PD$_x$ is rate controlling, i.e., $k_{-1} \gg k_2 + k_{-2}$. In Eq. [5], [P] is defined as the component in excess. If the scheme of Eq. [1] is operative, k_{obs} should go from a first-order dependence to a zero-

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[4] In derivation of Eq. [5], the first step is assumed to be fast so that the equilibrium ratio [PD$_i$]/[P][D] = K_1 is maintained throughout the reaction. Once this relationship is established, Eq. [5] can be derived in a manner analogous to that of Eq. [4].

order dependence on [P], as [P] increases from $[P] \ll 1/K_1$ to $[P] \gg 1/K_1$. Moreover, if there is an absorbance change associated with the formation of PD_i from P and D, the time dependence of the absorbance change should be biphasic, provided [P] is similar or greater than $1/K_1$. For example, if PD_i and PD_x have similar molar absorptivities, a substantial fraction of the absorbance change should occur very rapidly, perhaps within the dead time of the stopped-flow spectrometer, when $[P] \geq 1/K_1$. The previous stopped-flow studies of Gray and Glew (13) were conducted at a single temperature with the nitrophenyl glycoside in excess of the concanavalin A. At the high concentrations of chromogenic ligand necessary for their studies, the absorbance from excess ligand lowered the signal to noise ratio and also produced deviations from Beer's law due to stray light errors (13). This situation led to uncertainties in k_{obs} and prevented determination of the fraction of ligand binding which occurs during the slow process observed with the stopped-flow spectrometer. In the present studies, concanavalin A was the component in excess, thereby eliminating the background absorbance. Thus more accurate values of k_{obs} could be determined over a wider range of concentration of the component in excess. Also, the fraction of ligand binding associated with the slow process observed in the stopped-flow spectrometer could be determined.

Figure 1 illustrates the linear dependence of k_{obs} on the concentration of concanavalin A. The y-intercept of Fig. 1 is coincident with the first-order rate constant (k_{-a}) for the displacement of chromogenic ligand by a large excess of methyl α-D-mannopyranoside (L). At the concentrations used, the observed first-order rate constant (k_{-a}) for displacement was independent of the concentration of displacing ligand (20–100 mM), the concentration of chromogenic ligand (20–100 μM) and the concentration of concanavalin A combining sites (40–400 μM). For the scheme depicted in Eq. [1], displacement of chromogenic ligand from concanavalin A should occur via Eq. [9], when a solution containing protein and chromogenic ligand is mixed with displacing ligand.
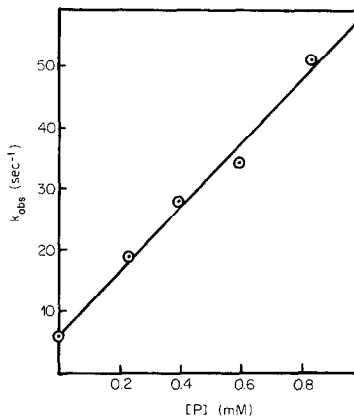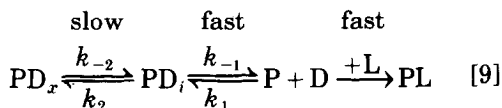

FIG. 1. Dependence of the rate constant k_{obs} on the concentration, [P], of concanavalin A combining sites after mixing at 25°C. The concentration of concanavalin A combining sites was 20–40 times the concentration of p-nitrophenyl α-D-mannopyranoside.

$$\text{PD}_x \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \text{PD}_i \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \text{P} + \text{D} \overset{+\text{L}}{\longrightarrow} \text{PL} \qquad [9]$$

$$\text{slow} \qquad \text{fast} \qquad \text{fast}$$

Provided all the protein is complexed with L at the end of the reaction, k_{-a} should equal k_{-2}. The identity of k_{-2} from a plot of Eq. [5] (Fig. 1) and k_{-a} verifies this equality provided the pathway of Eq. [1] applies. The linearity of the plot in Fig. 1 or the constancy of the value of k_a as determined from the relationship given in Eq. [2] can be used to set an upper limit for K_1. From Eq. [2], k_a is 5.5, 5.4, 4.7, and 5.4 × 10^4 s^{-1} M^{-1} at 233, 398, 598 and 831 μM concanavalin A combining sites. Assuming that the values of k_a are accurate to within 30%, K_1 must be smaller than 360 M^{-1}. Any value of K_1 larger than 360 M^{-1} would cause the values of k_a determined in the range 233–831 μM concanavalin A combinings sites to deviate from constancy by more than 30% at 831 μM concanavalin A (Eq. 6). With a value of $K_1 <$ 360 M^{-1}, no significant amount of PD_i exists in the concentration range of concanavalin A used. The following observations are consistent with this conclusion. (a) No evidence could be obtained for formation of PD_i in a rapid reaction occurring within the dead time of the stopped-flow spectrometer. The absorbance change appearing on the stopped-flow trace matched that of the difference in absolute absorbances of the

reactants and products determined in the stopped-flow spectrometer. (b) The change in molar absorptivity, $\Delta\epsilon$, on binding and displacement (2240 cm^{-1} M^{-1} and 1990 cm^{-1} M^{-1} at 25°C) as determined from the ratio of the change of absorbance on the stopped-flow trace to the amount of ligand bound (determined from the ratio k_a/k_{-a} and the initial concentration of reactants) was close to the value of 2020 ± 110 cm^{-1} M^{-1} determined from uv difference spectrometry. The values of $\Delta\epsilon$ determined from stopped-flow spectrometry were essentially independent of temperature between 9 and 37°C. Average values of 2320 ± 90 and 1940 ± 60 cm^{-1} M^{-1} were determined from ligand binding and displacement between 9 and 37°C. (c) The value of the overall association constant determined from kinetic measurements at 25°C, $K_a = k_a/k_{-a} = 0.87 \times 10^4$ M^{-1} agreed with the value of $1.1 \pm 0.1 \times 10^4$ M^{-1} determined from the dependence of the uv difference spectra of solutions of ligand and excess concanavalin A on the concentration of the protein.

The temperature dependencies of k_{-a} and k_a were determined. The rate constant k_a was calculated using Eq. [2], with k_{obs} determined at 233 μM concanavalin A combining sites. Figure 2 illustrates the linear relationships obtained between ln (k_a/T) or ln (k_{-a}/T) and $1/T$. These results indicate that in the temperature range studied, 9–37°C, $K_1 \leq 4300$ M$^{-1} = (1/233 \times 10^{-6}$ M), otherwise one would expect nonlinearity in the plot of (k_a/T) vs $1/T$, which was determined at 233 μM concanavalin A combining sites. The entropy of activation and enthalpy of activation for the binding of p-nitrophenyl α-D-mannopyranoside are listed in Table I. The entropies of activation listed in Table I for ligand binding and dissociation are much smaller than the values usually seen for changes in protein conformation. For example, the entropy of activation for the transition of α-chymotrypsin from a conformer which does not bind ligand to one which binds ligand is 29 entropy units (e.u.) (14). However, the large entropy changes associated with changes in the degree of hydration make it difficult to interpret entropy changes for reactions in aqueous solutions. The activa-
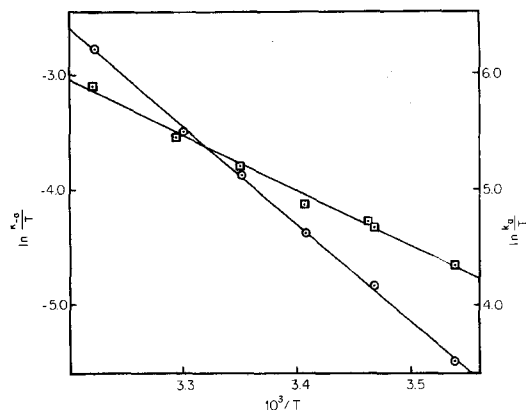

FIG. 2. Temperature dependence of k_a/T (□) and k_{-a}/T (○).

TABLE I

ACTIVATION PARAMETERS FOR THE ASSOCIATION AND DISSOCIATION OF THE COMPLEX BETWEEN CONCANAVALIN A AND p-NITROPHENYL α-D-MANNOPYRANOSIDE[a]

Temperature (°C)	$10^4 k_a$ (s^{-1} M^{-1})	Temperature (°C)	k_{-a} (s^{-1})
9.4	2.19	9.4	1.17
15.1	3.12	15.1	2.29
15.6	3.28	20.2	3.70
20.3	3.88	25.2	6.24
25.3	5.44	29.8	9.23
30.3	7.10	37.0	19.0
37.2	11.34		

$\Delta H^{\ddagger}_{(a)} = 9.5 \pm 0.3$ kcal/mol 0.3 $\Delta H^{\ddagger}_{(-a)} = 16.8 \pm 0.2$ kcal/mol

$\Delta S^{\ddagger}_{(a)} = -5.2 \pm 1.1$ e.u. 1.1 $\Delta S^{\ddagger}_{(-a)} = 1.3 \pm 0.7$ e.u.

$\Delta S^{\ddagger}_{u(a)} = 2.8 \pm 1.1$ e.u. $\Delta S^{\ddagger}_{u(-a)} = 1.3 \pm 0.7$ e.u.
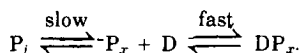
[a] pH 5.0, $\Gamma/2$ 0.5.

tion parameters for ligand binding to concanavalin A appear to be a function of the structure of the ligand. The rate constants at 25°C and the corresponding activation energies for the binding of the α- and β-isomers of methyl D-glucopyranoside, determined by Brewer et al. (8), are 5×10^4 s^{-1} M^{-1}, 5 kcal/mol and 2.8×10^4 s^{-1} M^{-1}, 2.5 kcal/mol, respectively. Using these values one obtains unitary entropies of activation for binding of the α- and β-isomers of methyl D-glucopyranoside of -14.3 and -23.9 e.u., respectively. These activation entropies are much larger than those

found with the chromogenic ligand; however, they do not require that ligand binding involve a large change in the conformation of concanavalin A.

The observation that carbohydrate ligands induce no change in the circular dichroic spectrum of concanavalin A below 240 nm (1, 2) suggests that an extensive structural rearrangement which alters the secondary structure of concanavalin A does not occur when carbohydrate ligands bind to concanavalin A.[5] The dependence of the entropy of activation for binding on ligand structure is also consistent with this view. However, some sort of substrate-induced change in the conformation of concanavalin A may be responsible for the low rate constants observed for formation of a protein–ligand complex. If binding of chromogenic ligand to concanavalin A is simply a one-step bimolecular process (Eq. [3]) the values of k_a and k_{-a} are equivalent to the rate constants for complex formation and decomposition, respectively. The value of $5.4 \times 10^4 \text{ s}^{-1} \text{ M}^{-1}$ (25°C) for the second-order rate constant for ligand binding differs substantially from values of about 10^9–$10^{10} \text{ s}^{-1} \text{ M}^{-1}$ expected for second-order rate constants for simple diffusion-controlled reactions (15). Usually, when second-order rate constants for ligand binding to a protein differ by so much from that expected for a diffusion-controlled reaction, a multistep pathway for ligand binding such as the one depicted in Eq. [1] is suspected.[6] The transition from PD_i to PD_x

is often visualized as a change in the conformation of the protein. Our failure to observe the intermediate state PD_i may be due to the equilibrium constant for its formation from P and D being less than 360 M^{-1}. However, it is also possible that the steric requirements for ligand binding are responsible for the low rate constants observed for ligand binding. For a ligand to bind to concanavalin A, it might have to undergo unfavorable steric interactions with one or more groups on the protein. These unfavorable interactions would be realized in the complex PD_i. Binding would be a simple one-step process (Eq. [3]) if the lifetime of PD_i corresponded to that of an activated complex.[7] Interestingly, the rate constants for ligand binding to most proteins are in the range 10^7–10^8 $\text{s}^{-1} \text{ M}^{-1}$ and are less than that expected for a diffusion-controlled reaction (16). Hammes and Schimmel (16) attributed these low rate constants to steric requirements for complex formation. Perhaps in some cases when the apparent rate constants for formation of protein–ligand complexes are substantially less than 10^7–10^8 $\text{s}^{-1} \text{ M}^{-1}$, binding is also a simple one-step bimolecular reaction involving unusually unfavorable steric interactions in the transition state. That steric factors can indeed lower second-order rate constants for formation of simple noncovalent complexes to values below that observed for formation of a concanavalin A–ligand complex was demonstrated by Cramer et al. (17) in their studies of the binding of dyes to α-cyclodextrins. Thus, further work is required to discover whether conformational changes and/or steric interactions are responsible for the low rates of formation of complexes

[5] The increased stability of ligand-bound protein against heat denaturation and proteolytic digestion can be explained without invoking an extensive ligand-induced change in conformation. Denaturation and digestion may well proceed through an intermediate consisting of partially unfolded protein which is at low concentration and in equilibrium with the properly folded protein. Ligand, by binding only the properly folded protein, would displace the equilibrium so as to lower the amount of partially unfolded protein and thereby lower the rate of denaturation and proteolytic digestion.

[6] Another pathway often associated with slow ligand binding is one involving a rate controlling change in conformation of the protein prior to ligand binding, i.e.,

$$P_i \underset{\text{slow}}{\rightleftharpoons} P_x + D \underset{\text{fast}}{\rightleftharpoons} DP_x.$$

The different activation parameters seen for different ligands and the dependence of the observed rate constant on the concentration of P eliminate this pathway for ligand binding to concanavalin A.

[7] If PD_i were an activated complex, its rate constant for decomposition to P and D would be $\sim kT/h$ and the equilibrium constant for formation of the activated complex K_1 might be low enough to cause the product $k_{-1}K_1$, i.e. k_1, to be much lower than the second-order rate constant for a diffusion controlled reaction. When PD_i is an activated complex, $k_{-1} = k_2$ and k_1 and k_{-2} of Eq. [1] become equivalent to k_a and k_{-a}, respectively.

between concanavalin A and simple carbohydrate ligands.

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