Binding Kinetics of Methyl α-D-Mannopyranoside to Concanavalin A: Temperature-Jump Relaxation Study with 4-Methylumbelliferyl α-D-Mannopyranoside as a Fluorescence Indicator Ligand†

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ABSTRACT: The binding of methyl α-D-mannopyranoside and methyl α-D-glucopyranoside to concanavalin A has been investigated by the temperature-jump relaxation kinetic technique using the competitive inhibitor 4-methylumbelliferyl α-D-mannopyranoside as an indicator of the binding reaction. The analysis shows that these saccharides bind to concanavalin A in a single bimolecular step. The binding parameters are compared to those of derivatized carbohydrates which have previously been used to study the binding of saccharides to concanavalin A. The similarity of the association rate constants indicates that a common process is involved in the binding of all carbohydrates to concanavalin A. The different affinities of saccharides for the lectin are primarily due to the different dissociation rate constants. A discussion of the proposed mechanism is given under the Appendix to clarify the fact that one of the observed relaxation times is faster than is possible with only the kinetic indicator reaction.

Binding of lectins to the carbohydrate moieties of cell wall glycoconjugates elicits a number of interesting biological responses, such as agglutination and mitosis. In addition, these proteins can differentiate between normal and malignant cells or between cells in different stages of development (Goldstein & Hayes, 1978; Lis & Sharon, 1977). Concanavalin A (Con A)[1] has been used extensively in this respect (Bittiger & Schnebli, 1976), and much work has been directed toward elucidating the details of carbohydrate binding to this protein. Methyl α-D-mannopyranoside (Me-Man*p*)[1] has been widely used as an effective competitive inhibitor in studies of carbohydrate-specific binding of the lectin concanavalin A to cell surface receptors (Bittiger & Schnebli, 1976; Goldstein & Hayes, 1978). It is therefore surprising that the thermodynamic binding parameters have been reported only recently (Munske et al., 1978; Van Landschoot et al., 1980a). These are augmented in the present study by using a competitive temperature-jump relaxation technique with the fluorescent 4-methylumbelliferyl α-D-mannopyranoside (MeUmb-Man*p*) as a well-characterized kinetic indicator (Loontiens et al., 1977a,b; Clegg et al., 1977) to obtain the rate parameters of Me-Man*p*. Due to kinetic coupling between the association reactions of the two ligands, two concentration-dependent relaxations are observed. An analysis of these relaxations yields the rate parameters and the molar enthalpy change for the binding of Me-Man*p* to the lectin. Similar competitive temperature-jump experiments show that the binding enthalpy of methyl α-D-glucopyranoside (Me-Glc*p*) is smaller than that of Me-Man*p*.

Experimental Procedures

The procedure for performing the relaxation measurements and the additional experimental conditions have been described previously (Clegg et al., 1977; Loontiens et al., 1977b). The temperature was increased by 3.2 °C to a final temperature of 23.2 °C. Free MeUmb-Man*p* was excited at 313 nm, and its fluorescence was detected through a 360-nm cutoff filter. No relaxations were observed in the absence of MeUmb-Man*p* or with any of the reaction components alone. All experiments were performed at pH 7.2 in 0.05 M Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 1 M NaCl, 1 mM $NiCl_2$, and 1 mM $CaCl_2$, with tetrameric concanavalin A composed of intact polypeptide chains (Con A). Protein concentrations were expressed on the basis of the subunit molecular weight of 25 500. The concentration of the competing ligand was varied (Me-Man*p* from 45 to 660 μM and Me-Glc*p* from 0.66 to 4.6 mM), keeping the total concentration of MeUmb-Man*p* (3.4 μM) and Con A (39 μM) constant. This concentration of protein results in a nearly maximal kinetic amplitude of the indicator reaction in the absence of any competing ligand. The kinetic data were stored directly in a DEC PDP 11/20 minicomputer and analyzed with a nonlinear regression program written by L. Avery (unpublished).

The concentration dependence of the relaxation times and amplitudes (species concentration changes) was simulated with a computer program which provides an exact solution for any reaction scheme studied by relaxation techniques (L. Avery, unpublished).

The results have been interpreted in terms of the model Scheme I

$$P + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PI \qquad K_1 = k_1/k_{-1} \tag{1a}$$

$$P + L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PL \qquad K_2 = k_2/k_{-2} \tag{1b}$$

where P = protein, L = fluorescent MeUmb-Man*p*, I = the competing ligand, and PL and PI are the complexes of Con A with MeUmb-Man*p* and competing ligand, respectively. The fluorescence of the indicator ligand L is totally quenched in the complex PL (Loontiens et al., 1977a). The competing ligand I does not fluoresce. Equilibrium concentrations of the reaction species[2] used for equilibrium binding analysis

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[1] Abbreviations used: Me-Man*p*, methyl α-D-mannopyranoside; Me-Glc*p*, methyl α-D-glucopyranoside; MeUmb-Man*p*, 4-methylumbelliferyl α-D-mannopyranoside; Con A, concanavalin A practically devoid of nicked polypeptide chain.

[2] The equilibrium concentration of reaction species is indicated by a line over the symbol for the species ($\bar{P}$, $\bar{L}$, etc.).

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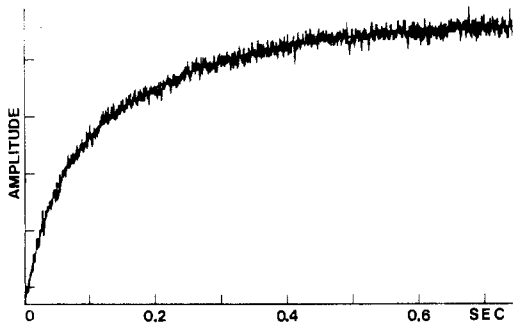

FIGURE 1: Biexponential temperature-jump relaxation curve of MeUmb-Man*p* fluorescence, obtained with 490 μM Me-Man*p* added to the indicator system (3.4 μM MeUmb-Man*p* and 39 μM Con A). The signal change is due to the increase of MeUmb-Man*p* fluorescence upon the dissociation of the totally quenched (MeUmb-Man*p*)–Con A complex. The biexponential fit corresponds to a faster relaxation (τ_f = 34 ± 1 ms, amplitude 18.6 ± 0.4 mV) and a slower relaxation (τ_s = 192 ± 3 ms, amplitude 32.1 ± 0.4 mV); the total amplitude is 3% of the static fluorescence (1696 mV). In the absence of Me-Man*p*, the relaxation curve was monoexponential (Clegg et al., 1977).

(Scatchard, 1949) were calculated according to this model from the static fluorescence readings in the temperature-jump cell as given in Van Landschoot et al. (1980a) by using the known value of the equilibrium constant for MeUmb-Man*p* (Loontiens et al., 1977a).

Results

Competition by Me-Manp. The static measurements of the MeUmb-Man*p* fluorescence yielded a linear Scatchard plot (not shown) for the binding of Me-Man*p* corresponding to 1.0 ± 0.1 binding site and an equilibrium association constant of (5.7 ± 0.4) × 10^3 M^{-1} for Me-Man*p* at 20 °C. The binding ratio, $\overline{PI}/\bar{P}$, for Me-Man*p* covered the relevant range from 0.2 to 0.8.

A typical temperature-jump relaxation is shown in Figure 1. It has been shown that only a single relaxation related to carbohydrate binding is observed with mixtures of MeUmb-Man*p* and Con A (Clegg et al., 1977; Loontiens et al., 1977b; see also Figure 3A, curve a). However, the addition of Me-Man*p* (I_0) to the above reaction system changes the observed relaxation spectrum. Two relaxations were always discernable at higher concentrations of Me-Man*p* (290–660 μM). At lower concentrations of Me-Man*p* the times of the fast (τ_f) and slow (τ_s) relaxations (Figure 2A) could not be reliably determined by the analysis, but the overall process became slower. The concentration dependence of the relaxation times and amplitudes is presented in Figure 2, and the results of the kinetic analysis, in terms of eq 1, are listed in Table I. The following features describe the data obtained in the presence of the competing ligand Me-Man*p*. (1) The faster relaxation is more rapid than the single relaxation observed with the MeUmb-Man*p*–Con A system alone (Clegg et al., 1977). This indicates a coupling of the MeUmb-Man*p*–Con A binding reaction to a faster process which involves Me-Man*p*. The amplitude of this faster relaxation process decreases with increasing $\bar{I}$ (Figure 2B), and $1/\tau_f$ increases (Figure 2A) as one would expect if this process were related closely to a bimolecular binding between Me-Man*p* and Con A. (2) The slower relaxation, which has the larger amplitude throughout, is slower than the single relaxation observed in the absence of I. This process becomes slower, and its amplitude decreases, as $\bar{I}$ increases. This suggests a decrease in the concentration of free protein binding sites ($\bar{P}$) available for the indicator $\bar{L}$.

The rate constants given in Table I refer to the model of Scheme I. The stimulated curves shown in Figure 2A,B, which

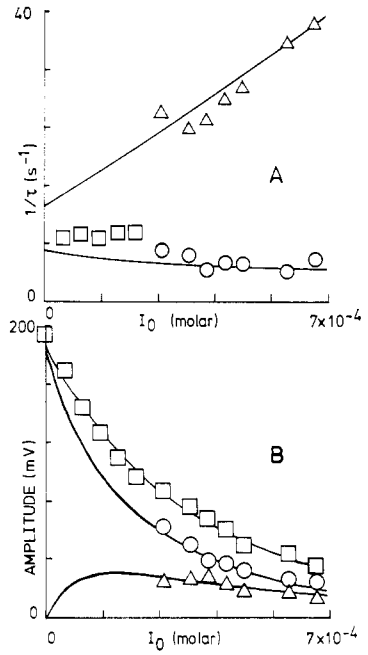

FIGURE 2: Plot of the inverse relaxation times (A) and amplitudes (B) as a function of total Me-Man*p* (I_0) concentration. The slow (○) and fast (△) relaxation times and amplitudes can only be separated above I_0 = 290 μM. (□) refers to the apparent single relaxation time below I_0 = 290 μM and also to the total kinetic amplitude throughout the whole concentration range. The experiments were done with fixed concentrations of the indicator system (3.4 μM Me-Man*p* and 39 μM Con A). The solid curves were calculated according to the generalized rate expressions for Scheme I as explained under Experimental Procedures by using the reaction rate parameters given in Table I and $\Delta H°$ = −8.5 kcal/mol for the binding of MeUmb-Man*p* (Clegg et al., 1977). This yielded an optimal value of $\Delta H°$ = −9.7 kcal/mol for the binding of Me-Man*p*.

Table I: Comparison of Reaction-Rate Parameters and Kinetically Defined Association Constants for Binding of Carbohydrates to Con A according to a Bimolecular One-Step Association[a]

	$10^{-4} \times k_1$ (M^{-1} s^{-1})	k_{-1} (s^{-1})	$10^{-3} \times K$ (M^{-1})	t (°C)	pH
MeUmb(Man*p*)$_2$[b]	2.8	0.12	240	20	5.5
	3.6	10.1	35	20	5.5
p-NO_2Ph(Man*p*)$_2$[c]	3.2	0.22	145	25	5.0
	2.3	1.1	21	25	5.0
MeUmb-Man*p*[d]	10.2	3.2	32	24.1	7.2
	11.3	3.4	33	24.1	5.5
p-NO_2Ph-Man*p*[e]	5.4	6.2	8.7	25	5.0
p-NO_2Ph-2-*O*-Me-Man*p*[f]	6.6	8.2	8.1	25	5.0
Me-Man*p*[g]	4	12	3.3	23.2	7.2
Me-Glc*p*[g,h]	5	31	1.7	25	7.2[g], 5.6[h]
Me-β-Glc*p*[h]	2.8	400	0.07	25	5.6

[a] The data for MeUmb(Man*p*)$_2$, *p*-NO_2Ph(Man*p*)$_2$, *p*-NO_2Ph-Man*p*, and *p*-NO_2Ph-2-*O*-MeMan*p* were obtained by stopped-flow kinetics and for MeUmb-Man*p* by temperature-jump relaxation. The two sets of data for the mannobiosides reflect two binding modes. [b] 4-Methylumbelliferyl α-mannobioside (Van Landschoot et al., 1980b). [c] *p*-Nitrophenyl α-mannobioside (Williams et al., 1978). [d] Clegg et al., 1977; Loontiens et al., 1977b. [e] *p*-Nitrophenyl α-D-mannopyranoside (Lewis et al., 1976). [f] *p*-Nitrophenyl 2-*O*-methyl α-D-mannopyranoside (Williams et al., 1978). [g] Competitive temperature-jump relaxation (this work). [h] Spin-lattice ^{13}C NMR relaxation using Me-Glc*p* and methyl β-D-glucopyranoside (Me-β-Glc*p*) (Brewer et al., 1973, 1974).

are produced from the complete expressions for the general model, show that the results are consistent with a one-step bimolecular association of I to Con A. By use of the known

change in enthalpy for the binding of MeUmb-Man*p* (Loontiens et al., 1977a) of $\Delta H^\circ = -8.5$ kcal/mol, the ΔH° of binding for Me-Man*p* was determined to be −9.7 kcal/mol.

Competition by Me-Glcp. The Scatchard plot (not shown) obtained from the static fluorescence readings was linear throughout the examined range of the binding ratio $\overline{PI}/\bar{P} = 0.5$–$0.9$ for Me-Glc*p*. The resulting equilibrium association constant for Me-Glc*p* was $(2.1 \pm 0.1) \times 10^3$ M^{-1} at 20 °C and corresponded to a single binding site per protomer. This affinity constant is in good agreement with 1.7×10^3 M^{-1} at 25 °C, determined kinetically by Brewer et al. (1974).

The results of the kinetic experiments (Figure 3) are similar to those of the corresponding experiments with Me-Man*p* except that the faster relaxation could be resolved only within a very narrow range of $\bar{I}$. The kinetic coupling between the two reactions of eq 1 must be minimal since the small amplitude of the faster relaxation never exceeded 4% of the total amplitude. The reaction rate parameters for Me-Glc*p* as given in Table I (Brewer et al., 1974; predict the observed relaxation time (12 ± 2 ms) satisfactorily to be 15.7 ms.

The behavior of the relaxation parameters of the slower process is shown in Figure 3C1,C2. A plot of the inverse of this relaxation time vs. the sum of free concentrations of Con A binding sites and MeUmb-Man*p* ($\bar{P} + \bar{L}$) is linear (not shown). If the slope and intercept of this plot are interpreted as the rate constants for a bimolecular reaction (Scheme Ib, eq 2 of Discussion and Appendix) we have $k_2 = (1.2 \pm 0.3) \times 10^5$ M^{-1} s^{-1}, $k_{-2} = (2.8 \pm 0.1)$ s^{-1}. These values are in excellent agreement with the rate constants for MeUmb-Man*p* in Table I.

The change in the enthalpy of binding of Me-Glc*p* with Con A, eq 1b, can be estimated from the data in Figure 3C2 ($\Delta H^\circ = -4.3$ kcal/mol). The equilibrium association constant can be determined from the slower relaxation times as described in Figure 3B and by eq A5, A7, and A9 (see Appendix).

Discussion

Previous kinetic studies of carbohydrate–Con A interactions have used chromophoric derivatives such as aryl α-D-mannopyranosides (Gray & Glew, 1973; Williams et al., 1978; Clegg et al., 1977; Loontiens et al., 1977b) and aryl α-mannooligosaccharides (Williams et al., 1978; Van Landschoot et al., 1980b) to provide convenient optical parameters. In order to investigate the kinetics of methyl glycosides, which produce no significant optical change, we have used MeUmb-Man*p* as an indicator of the binding reaction. The advantages of MeUmb-Man*p* for this purpose are as follows: (a) complete quenching of fluorescence upon binding (Dean & Homer, 1973; Loontiens et al., 1977a), (b) a simple binding mechanism (Clegg et al., 1977; Loontiens et al., 1977b), and (c) a binding which can be completely inhibited by simple glycosides and mannooligosaccharides (Loontiens et al., 1977a; Van Landschoot et al., 1980a).

A kinetic indicator reaction is normally chosen such that its specific reaction is much faster than the reaction under investigation (Bernasconi, 1976). This restriction is not necessary since a faster process can be observed through sufficient kinetic coupling to a slower indicator reaction. We observe an additional relaxation upon adding Me-Man*p* to a solution containing Con A and MeUmb-Man*p* which is faster than the indicator system (MeUmb-Man*p*–Con A) alone. As shown below the simple mechanism of Scheme I is sufficient to account for the data, and the rate constants for the binding of Me-Man*p* to Con A can be determined.

We have analyzed our results in terms of Scheme I, and the experimental results have been simulated (Figure 3C1,C2) by

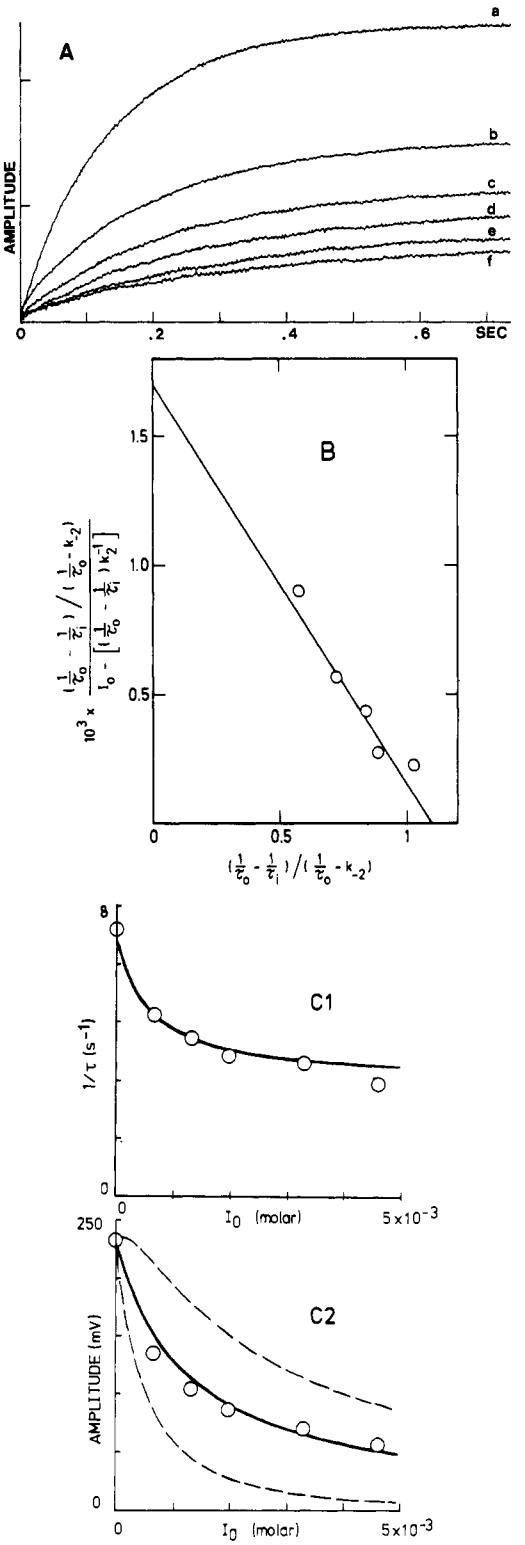

FIGURE 3: (A) Temperature-jump relaxation obtained with the indicator system of MeUmb-Man*p* and Con A in the presence of different concentrations (mM) of Me-Glc*p*: (a) 0; (b) 0.66; (c) 1.31; (d) 1.97; (e) 3.29; (f) 4.6. The trace is monoexponential ($\tau = 135$ ms) when Me-Glc*p* is absent, and the amplitude and time decrease as the Me-Glc*p* concentration increases. Con A and MeUmb-Man*p* concentrations are as given in Figure 1. The biexponential character in the presence of Me-Glc*p* could only be resolved when [Me-Glc*p*] = 0.66 mM (see text). (B) Scatchard plot for the binding of Me-Glc*p* constructed from the slow relaxation time (see Appendix). As with a normal Scatchard plot the slope gives the equilibrium binding constant if the number of binding sites is one. (C) Plot of the relaxation time (1) and amplitude (2) for the slow relaxation as a function of Me-Glc*p* concentration. The smooth curve was obtained from the rate parameters for MeUmb-Man*p* (Table I) and the equilibrium constant for Me-Glc*p* (B); the fit was optimal with $\Delta H^\circ = -4.3$ kcal/mol for Me-Glc*p*. The dashed curves indicate simulations with $\Delta H^\circ = 0$ and −9 kcal/mol to show the accuracy of the determination.

using the general formalism of this model. However, the expressions for relaxation times and amplitudes can be simplified under conditions applicable to our case (see Appendix) so as to reveal the essential features of the observed reaction process.

$1/\tau_s$ decreases in a hyperbolic manner, analogous to a binding isotherm (Figure 2), as the concentration of the simple saccharide, $\bar{I}$, increases. This would be expected if the effect of the methyl glycoside, I, were restricted to a competitive inhibition of the binding of MeUmb-Man*p*, L, thereby only decreasing the free Con A sites available for the binding of $\bar{L}$. In the absence of appreciable kinetic coupling (see Appendix) the slow relaxation time is

$$1/\tau_s = k_2(\bar{P} + \bar{L}) + k_{-2} \quad (2)$$

If $\bar{P} \gg \bar{L}$, then $1/\tau_s$ provides a direct measurement of $\bar{P}$ (see eq A7a of Appendix). Thus a determination of the equilibrium constant of the methyl glycoside, I, is possible using only the slow relaxation times and the known rate constants of the MeUmb-Man*p* reaction (see Figure 3B and Appendix). For Me-Glc*p*, the two relaxation processes are well separated for all values of $\bar{I}$, and the estimate of the binding constant from this analysis agrees very well with a previous kinetic determination (Brewer et al., 1974) and with the static fluorescence results (see Results). This type of analysis for Me-Man*p* yields an equilibrium constant which is in fairly good agreement with the general analysis even though the relaxation times are not always well separated. Therefore, the behavior of the slow relaxation time is dominated by the MeUmb-Man*p* reaction. It certainly cannot be directly related to a bimolecular binding reaction with methyl glycosides since τ_s would then decrease as a function of $\bar{I}$.

The faster relaxation process directly demonstrates the coupling of the two reactions of Scheme I since the relaxation time is faster than with the indicator, L, alone and there is no signal change associated with the binding of the methyl glycoside, I, to Con A. If we assume that the faster process involves only the bimolecular association of Me-Man*p* to Con A, we can estimate an equilibrium constant by analyzing the data according to eq A2a. This analysis gives 3.3×10^3 M^{-1} which agrees well with the value determined from the static fluorescence measurements. In addition, these rate constants derived from the simplified analysis agree within the experimental error with the estimates from the general formalism (Table I, Figure 2A). Thus we are justified in interpreting the faster relaxation time (not the amplitude) as though it is associated with an independent reaction.

The present study provides an example of the interesting fact that the expressions for the relaxation times are well approximated without considering kinetic coupling, although the observable amplitude of the faster process can only arise from the kinetic coupling of the two competitive reactions. The relaxation amplitudes appear more sensitive to this coupling especially since the reference value for the amplitude of the uncoupled faster reaction (Scheme I, eq 1a) is 0 (see eq A3 of Appendix). This dichotomy between the relaxation times and amplitudes is exemplified by the fact that the two relaxation times are well separated at the higher concentrations of Me-Man*p*, although the observable amplitude of the faster process is only slightly lower than that of the slower process.

Table I lists the rate constants for the binding of Con A to several carbohydrates including Me-Man*p*. It is clear that the large aryl groups of the carbohydrate derivatives have no profound kinetic effect. These data together with those of Farina & Wilkins (1980) permit two important generalizations: (1) the forward rate constants are similar, and much lower than diffusion-controlled rates; (2) the dissociation rates vary considerably and reflect the relative affinities of the ligands. These properties may apply to all saccharide interactions with Con A. Other carbohydrate–protein systems with low association rates include antibody–hapten complexes (Pecht & Lancet, 1977) such as anti-lactose and anti-dextran IgM, homogeneous and heterogenous hybrid IgA's with galactan specificity (Zidovetzki et al., 1980), lysozyme (Chipman & Schimmel, 1968), the lectin from *Ricinus communis* (Podder et al., 1978), wheat germ agglutinin (Clegg et al., 1980), and Pneumococcal antibody (Maeda et al., 1977).

The association rate constants of many of the above systems are far smaller than expected for a diffusion-controlled reaction, and this difference cannot be easily explained by the type of steric arguments which have been invoked to account for the decreased rates of many protein–ligand systems (Hammes & Schimmel, 1970). In the case of carbohydrates which bind to Con A, the observed association rate is 4 orders of magnitude lower than that calculated for a diffusion-controlled reaction which would place very severe requirements on steric constraints (i.e., frequency factors). Including the effect of rotational diffusion (Schmitz & Schurr, 1972) would not yield such a large reduction due to the fast rotational diffusion of the ligands.

A general phenomenon which could reduce the apparent association rates of any bimolecular system is the putative existence of a very rapid isomerization of one of the reactive components such that only a minor species would react. However, dilution kinetic experiments with MeUmb-Man*p* and Con A (Clegg et al., 1977) have definitely excluded the possibility that only a minor species of either protein or ligand is involved. Brewer et al. (1974) have also shown that the methyl α- and β-D-glucopyranosides bind to Con A in the $^4C_1$ conformation which also dominates in solution. Most likely this conclusion about MeUmb-Man*p* can be extended to interactions of other carbohydrates with Con A.

The slow association rates of all carbohydrates and Con A (Table I) are consistent with a reaction step with a high activation energy. In addition, the fact that the association rates are all very similar suggests a rate-controlling process which is common for the association of all the ligands with Con A. A conformational change of the protein–ligand encounter complex has been previously considered (Lewis et al., 1976; Clegg et al., 1977) in order to define consistent limits for the equilibrium constant of an encounter complex. However, it should be emphasized that there is no direct kinetic evidence of such a change in the fully metallized form of the protein upon the binding of a carbohydrate. A conformational change in Con A upon interaction with a carbohydrate has been postulated from CD (Pflumm et al., 1971; Cardin & Behnke, 1979), from NMR stopped flow (Grimaldi & Sykes, 1975) and from the fact that crystals of Con A will crack when infused with Me-Man*p* (Hardman & Ainsworth, 1973). It has also been inferred that any change of the protein structure upon the binding of a carbohydrate does not alter the geometry of the metal binding sites (Nicolau et al., 1969; Richardson & Behnke, 1976). CD studies indicate that any conformational changes do not involve the secondary structure of the protein but are either local or related to the tertiary structure (Cardin & Behnke, 1979). The kinetic results in Table I and the above evidence for an isomerization of the protein suggest that a conformational change [within the limits of the bimolecular step discussed in Clegg et al. (1977)] plays an important role in the specificity of carbohydrate recognition by Con A. Such a change does not seem to involve large overall

movements of the protein structure and may be rather local. Such restricted processes may involve the removal of tightly bound water or hydrogen-bond interactions in the protein which are replaced or augmented by the carbohydrate. Such a process is reasonable from a kinetic point of view since the dissociation rate constants of Table I vary considerably, and this might not be expected if a conformational change involved only (or mainly) the protein structure. The widely differing reverse rate constants reflect the differential affinity of the carbohydrate–Con A complex. In this respect it is important to note that the enthalpy of binding for α-D-mannopyranosides is on the order of −8.5 to −9.0 kcal/mol (Van Landschoot et al., 1978, 1980a). This is considerably different from that for Me-Glc*p* (ΔH° = −4.3 kcal/mol), and this could reflect the different degree of hydrogen bonding for both carbohydrates. A comparison of ΔH° values for carbohydrate binding to Con A given by several authors (Farina & Wilkins, 1980) would be difficult in view of the possible influence of the ionic strength on the observed thermodynamic parameters (Munske et al., 1978).

An important conclusion of this work is that the association rate for Me-Man*p* binding to Con A is very similar to those found for the *p*-nitrophenyl- and MeUmb-Man*p*–mannosides and that the overall mechanism of binding is not affected by the chromophoric aglycons of these derivatives.

Acknowledgments

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Appendix

The following treatment of the relaxation kinetic equations is based upon the formalism presented by Castellan (1963). The change in concentration of species i present in solution is related to the change in advancement of each reaction ($\Delta\epsilon^\alpha$) by the relationship

$$\Delta c_i = (\sum_\alpha \nu_{i\alpha}\Delta\epsilon^\alpha)/V$$

V is the total volume, $\nu_{i\alpha}$ is the stoichiometric coefficient of species i in the αth reaction, and the time-dependent deviation of the net advancement of the αth reaction from its equilibrium value is $\Delta\epsilon^\alpha = \epsilon^\alpha - \epsilon_0{}^\alpha$ ($\epsilon_0{}^\alpha$ = equilibrium advancement of the αth reaction). This variable will be referred to as the change in advancement of the αth reaction. Using the rules formulated by Castellan (1963) the differential equations which define the relaxation times and amplitudes for any reaction mechanism are easily derived. For the reaction mechanism of Scheme I in the main text, the matrix form is

$$\frac{d}{dt}\begin{bmatrix}\Delta\epsilon^1\\ \Delta\epsilon^2\end{bmatrix} = \begin{bmatrix}k_1(\bar{P}+\bar{I})+k_{-1} & -k_1\bar{I}\\ -k_2\bar{L} & k_2(\bar{P}+\bar{L})+k_{-2}\end{bmatrix}\begin{bmatrix}\Delta\epsilon^1\\ \Delta\epsilon^2\end{bmatrix} \quad \text{(A1)}$$

All notations are the same as given in the text.

Expressions for Well-Separated Relaxation Times. The eigenvalues of the matrix in eq A1 are the inverse relaxation times for the reaction scheme, and provided that the first reaction equilibrates much faster than the second reaction (kinetic uncoupling)

$$(1/\tau)_f = k_1(\bar{P}+\bar{I}) + k_{-1} \quad \text{(A2a)}$$

$$(1/\tau)_s = k_2(\bar{P}+\bar{L}) + k_{-2} - k_1k_2(\bar{I})(\bar{L})/[k_1(\bar{P}+\bar{I}) + k_{-1}] \quad \text{(A2b)}$$

The subscripts f and s refer to fast and slow, respectively. If the concentrations are such that $\bar{L} \ll \bar{P}, \bar{I}$, then the last term of eq A2b is insignificant and $(1/\tau)_s$ becomes eq 2 under Discussion. This means that the expression for each relaxation time involves only the rate constants of one of the reactions and the coupling occurs only through the equilibrium concentrations of the species [see also p 34 of Bernasconi (1976)].

Amplitudes and Their Dependence upon Kinetic Coupling of the Two Competing Reactions of Scheme I. Although it may be reasonable to analyze the relaxation time data as though the time course of the two reactions were completely independent, kinetic coupling must be considered to analyze even approximations to the amplitudes. The expressions for the observed relaxation amplitudes are found by solving for the eigenvectors of the matrix in eq A1 and then imposing the thermodynamic boundary conditions. However, the coupling of the slower reaction [eq 2 under Discussion] to the faster relaxation is most clearly demonstrated by considering the eigenvectors themselves.

We define the eigenvector of the matrix in (A1) corresponding to the faster relaxation to be $(\Delta\epsilon_f{}^1, \Delta\epsilon_f{}^2)$, where $\Delta\epsilon_f{}^i$ is the ith component of the normalized eigenvector of the change in advancement (for the ith reaction). The ratio of the eigenvector components is

$$\frac{\Delta\epsilon_f{}^2}{\Delta\epsilon_f{}^1} = \frac{-(1/\tau)_f + k_1(\bar{P}+\bar{I}) + k_{-1}}{k_2\bar{I}} \quad \text{(A3)}$$

By substituting the expression of (A2a) into (A3), this ratio becomes 0, which is expected since (A2a) was derived by assuming that the rates of reaction a of Scheme I are sufficiently fast to cancel any kinetic coupling between the two reactions. It can be shown that at higher $\bar{I}$ the first-order corrected expression for $(1/\tau)_f$ which includes coupling of the two reactions is

$$(1/\tau)_f = k_1(\bar{P}+\bar{I}) + k_{-1} - k_2\bar{L} \quad \text{(A4)}$$

Since $\bar{L}$ is kept very low in all experiments the last term of eq A4 will not contribute significantly to the value of the relaxation time. However, the ratio of eq A3 now becomes finite and is

$$\frac{\Delta\epsilon_f{}^2}{\Delta\epsilon_f{}^1} = \frac{k_2\bar{L}}{k_1\bar{I}} \quad \text{(A5)}$$

Thus the change in advancement of the slower reaction is finite even though the faster relaxation *time* appears to be unaffected by the slower reaction. Equation A5 shows that the change in advancement for the indicator reaction (which is slower) will contribute to the overall change in advancement for the faster relaxation process by a fractional amount which is similar to the fractional error in the relaxation time (the coupling term is $k_2\bar{L}$ in both cases). The end effect upon the measured amplitude depends upon the relative change in the molar enthalpies of the two reactions, their equilibrium constants, and the contribution of each reaction to the measured parameter. Once expressions for the eigenvectors have been derived, the observed amplitudes can be easily written down by using the formalism developed by Jovin (1975). In the present work, this small kinetic coupling is responsible for the fact that the two measured amplitudes are almost equal for the higher concentrations of Me-Man*p*.

Equilbrium Binding Analysis Using Only Slow Relaxation Times. We derive here a method of analysis, similar to a normal Scatchard plot, for finding the equilibrium constant of the first (faster) reaction of Scheme I of the text by using only the relaxation times of the slower relaxation. We assume that $\bar{L}$ (and therefore $\overline{PL}$) is insignificant, and so

$$\overline{PI} = \bar{P}_0 - \bar{P} \quad \text{(A6a)}$$

$$\bar{I} = \bar{I}_0 - \overline{PI} \quad \text{(A6b)}$$

According to eq 2 (see Discussion), we can write

$$\bar{P} = (1/k_2)[1/(\tau)_s - k_{-2}] \quad \text{(A7a)}$$

$$\bar{P}_0 = (1/k_2)[1/(\tau_0)_s - k_{-2}] \quad \text{(A7b)}$$

The quantities $\bar{P}_0$ and $\bar{I}_0$ refer to the respective analytical concentrations, and $(\tau_0)_s$ is the slow relaxation time in the absence of I. Using the relationships (A6a), (A6b), (A7a), and (A7b), we can write an expression for the "fraction of bound protein sites", FB, in terms of the relaxation time of the slow process

$$\text{FB} = (\text{bound sites})/(\text{total sites}) = \overline{PI}/\bar{P}_0$$

$$\text{FB} = [1/(\tau_0)_s - 1/\tau_i]/[1/(\tau_0) - k_{-2}] \quad \text{(A8)}$$

where $1/\tau_i$ is the relaxation time for the ith $\bar{I}$. And

$$\bar{I} = \bar{I}_0 - [1/(\tau_0)_s - 1/\tau_i]/k_2 \quad \text{(A9)}$$

A Scatchard plot, $\text{FB}/\bar{I}$ vs. FB, may be constructed by using eq A8 and A9 where the experimental quantities are relaxation times. This simple approach has the advantage that only the relaxation times (see Figure 3B) and the well-known kinetic constants of the indicator reaction are used to calculate the equilibrium constant of the unknown ligand. Thus instrumental instabilities which interfere with either static fluorescence or amplitude measurements are avoided.

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