Carbon-13 Nuclear Magnetic Resonance Studies on Lectin–Carbohydrate Interactions: Binding of Specifically Carbon-13-Labeled Methyl β-D-Lactoside to Peanut Agglutinin†

Klaus J. Neurohr,* Henry H. Mantsch, N. Martin Young, and David R. Bundle

ABSTRACT: The binding of methyl β-D-lactoside, specifically ^{13}C-labeled at C-1 of the D-galactose residue, to peanut agglutinin was studied by carbon-13 nuclear magnetic resonance. The high level of ^{13}C enrichment and the high magnetic field employed allowed studies at low ligand concentrations and provided simple single line spectra of very good signal to noise ratios. The ^{13}C-1 resonance of the disaccharide shows line broadening upon binding to the lectin, which was inhibited by an excess of an unlabeled competitive binding sugar. Between 10 and 30 °C, the disaccharide was found to be in slow exchange with the protein. Residence times, τ_M, dissociation rate constants, k_{diss}, and association equilibrium constants were obtained from a study of the ^{13}C-1 line width of the disaccharide in the presence of the protein. Dissociation rate constants for the disaccharide were similar in magnitude to those found for the α and β anomers of methyl D-galactoside but showed a steeper temperature dependence. The activation enthalpy for dissociation, 13.4 kcal mol^{-1}, is larger than that for methyl β-D-galactopyranoside, while the activation entropy is less negative. The free energy of activation for dissociation is very close to that for both methyl α- and β-D-galactopyranoside. The association rate constants for the disaccharide at different temperatures, as calculated from the dissociation rate constants and from association constants determined independently, are also very similar to those found for the monosaccharide. The association activation parameters, $\Delta H^* = -3.5$ kcal mol^{-1} and $T\Delta S^* = -14.6$ kcal mol^{-1}, and the fact that the activation enthalpy for dissociation is smaller than the total enthalpy change are further evidence for the previously proposed two-step model for the binding of saccharides to peanut agglutinin. The larger dissociation activation enthalpy and total enthalpy change on binding of the disaccharide, as compared to the monosaccharide, indicate an extended binding site on peanut agglutinin.

The specific recognition of carbohydrates by proteins is a critical event in many biological processes involving the cell surface (Hughes & Sharon, 1978). It is therefore desirable to establish the physicochemical details of the interactions between carbohydrates and proteins. For this purpose, lectins offer particularly useful examples of carbohydrate-recognizing proteins amenable to studies by physical techniques. Although their physiological roles are not entirely defined, they are probably involved in cell–cell interactions; for example, in plants they may play a role in the symbiosis with nitrogen-fixing bacteria. The lectins specifically bind simple and complex carbohydrates and are widely employed as cell-surface probes (Goldstein & Hayes, 1978). By use of monosaccharide and disaccharide derivatives with spectroscopic reporter groups such as 4-methylumbelliferyl and *p*-nitrophenyl, the binding kinetics of concanavalin A (con A)[1] and the lectin from *Ricinus communis* have been determined (Clegg et al., 1977; Podder et al., 1978; van Landschoot et al., 1980a; Farina & Wilkins, 1980). ^{13}C NMR spectroscopy offers the advantage over these approaches in that bulky external labels are not required, and it has been well established as a powerful nonperturbing technique. NMR studies of ligands at ^{13}C natural abundance, however, are hampered by the relatively low detection sensitivity of ^{13}C, the background spectrum from the protein, and the possible overlap of resonances. Specific ^{13}C labeling of ligand carbon atoms provides a decided advantage for such studies. In the case of carbohydrates, specific ^{13}C labeling of the C-1 position of monosaccharides is synthetically feasible (Serianni et al., 1979).

We have used these approaches to study the binding kinetics of methyl galactosides to the anti-T lectin from peanuts, employing specifically labeled methyl α- and β-D-galactopyranoside (Neurohr et al., 1981). For both ligands, association and dissociation rate constants were similar in magnitude to those found for the binding of sugars by con A. A considerable activation entropy was found for the association process but essentially no activation enthalpy. A two-step binding model was proposed, consistent also with CD and UV spectroscopic data (Neurohr et al., 1980), that involves a conformational change of the protein upon sugar binding.

To investigate differences in the thermodynamics and kinetics of the binding of larger saccharides compared to the simple monosaccharides, we extended our studies to the disaccharide methyl β-D-lactoside. Chemical synthesis provided this ligand specifically ^{13}C labeled at C-1 of the D-galactose residue, and this disaccharide glycoside is the first elaborate saccharide to be used for such studies. Reaction rate constants and activation parameters from these studies reveal differences for the binding of the larger saccharide, suggesting an extended binding site on peanut agglutinin.

Experimental Procedures

Materials. PNA was purified from fresh peanuts by affinity chromatography on a lactose-based affinity matrix as described previously (Neurohr et al., 1980). The lectin was eluted from the column with 0.1 M D-galactose in PBS. Protein concen-

† From the Division of Chemistry and the Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A 0R6. *Received June 30, 1981.* This paper is NRCC Publication No. 18922. K.J.N. was supported by a fellowship from the Deutsche Akademische Austauschdienst. This work is part of the Ph.D. thesis of K.J.N.

[1] Abbreviations: methyl β-D-lactoside, methyl 4-*O*-β-D-[1-^{13}C]-galactopyranosyl-β-D-glucopyranoside; PNA, peanut agglutinin; con A, concanavalin A; PBS, phosphate-buffered saline (0.01 M phosphate and 0.15 M NaCl, pH 7.2); ^{13}C NMR, carbon-13 nuclear magnetic resonance; CD, circular dichroism; ^1H NMR, proton nuclear magnetic resonance; Me_4Si, tetramethylsilane.

0006-2960/82/0421-0498$01.25/0

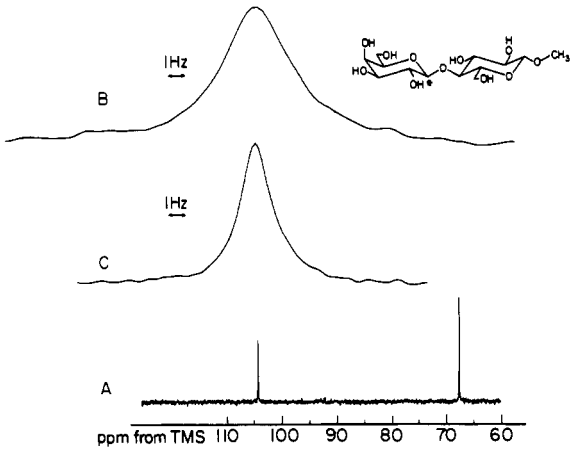

FIGURE 1: (A) Proton-decoupled ^{13}C NMR spectrum of 9×10^{-4} M methyl β-D-lactoside (90% ^{13}C at C-1 of the D-galactose residue) in the presence of 9.2×10^{-5} M PNA tetramers in PBS at pH 7.2 and 30 °C. (B) is the same spectrum shown on an expanded scale, and (C) is the spectrum observed after addition of 30 mM unlabeled methyl α-D-galactopyranoside, a competitive binding sugar. 1-Hz digital line broadening was applied in (B) and (C). The resonance at high field in (A) is due to the *p*-dioxane internal standard. The asterisk in the structural formula of methyl β-D-lactoside indicates the position of the ^{13}C label.

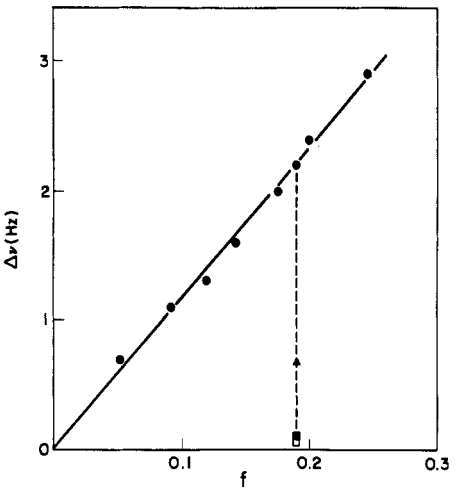

FIGURE 2: Change in C-1 line width ($\Delta\nu$) of ^{13}C-labeled methyl β-D-lactoside in the presence of 9.2×10^{-5} M PNA tetramers as a function of *f*, the fraction of sugar bound at 30 °C. Spectra were obtained in PBS at pH 7.2. The dashed line indicates the line-width change in a sample containing 9×10^{-4} M methyl β-D-lactoside to which was added 10 (▲), 20 (■), and 30 mM (□) unlabeled methyl α-D-galactopyranoside.

trations were determined by absorption with $A_{280}^{1\%} = 7.7$ (Lotan et al., 1975). The homogeneity of the lectin was established by polyacrylamide gel electrophoresis. Methyl β-D-lactoside, specifically ^{13}C labeled at the C-1 position of the D-galactose residue, was synthesized by a Koenigs–Knorr reaction between suitably activated and protected derivatives of [1-^{13}C]-D-galactose and methyl β-D-glucopyranoside. The details of this multistep synthesis are the subject of a separate publication.[2] Anomeric purity of the labeled methyl β-D-lactoside was established by ^1H and ^{13}C NMR. These, in conjunction with optical rotation and analytical data, confirmed the identity and purity of the ligand.

NMR Measurements. NMR samples were made up in PBS buffer at pH 7.2 containing 0.02% sodium azide. The protein concentration was 6.5×10^{-5} or 9.2×10^{-5} M in PNA tetramer. Experiments were carried out with three different batches of PNA, which were freshly prepared for the NMR measurements. The concentration of the ^{13}C-labeled disaccharide varied from 6×10^{-3} to 4×10^{-4} M. *p*-Dioxane was used as an internal standard. All samples contained 20% D_2O for field frequency locking of the spectrometer. ^{13}C NMR spectra were recorded in tubes of 10-mm diameter on a Bruker CXP-300 spectrometer at 75.5 MHz with quadrature detection and broad-band proton decoupling. The temperature of the probe was maintained with a Bruker BVT 1000 temperature unit and monitored with a thermocouple in the probe. Line widths measured at half-height of the ^{13}C resonance of the enriched carbon of the disaccharide were corrected for magnetic field inhomogeneity by using the half-width of the *p*-dioxane resonance.

Results

Effect of Protein Binding on the C-1 Line Width of Methyl β-D-Lactoside. Figure 1 shows a ^{13}C NMR spectrum of methyl β-D-lactoside, ^{13}C labeled at the C-1 position of the D-galactose residue, in the presence and absence of peanut agglutinin. The chemical shift of the C-1 resonance at 25 °C in PBS buffer at pH 7.2 was found at 36.4 ppm, relative to internal *p*-dioxane (104.1 ppm relative to external Me_4Si). In the presence of the lectin, the C-1 resonance shows line broadening, as displayed in Figure 1, but no change in chemical shift in the temperature range of 5–30 °C. A variety of experiments were carried out to ascertain that the observed line-broadening effects are due to specific binding of the disaccharide to the lectin. At a constant temperature, the change in line width $\Delta\nu$ of the C-1 resonance of the disaccharide was linear with the fraction *f* of sugar bound to the protein as shown in Figure 2 for 30 °C. The fractions bound for different sugar concentrations, at a constant PNA concentration, were calculated from the association constants as obtained from NMR in this study (see below). The total sugar concentration [S], at constant PNA concentration and temperature, is a linear function of the reciprocal change in line width, $1/\Delta\nu$, when plotted according to eq 1 (see below). Moreover, the K_D values obtained from the *y* intercepts of these plots at different temperatures are in excellent agreement with those determined independently by UV difference spectroscopy.[3] Upon addition of an excess of unlabeled methyl α-D-galactopyranoside, a competitive binding sugar (Neurohr et al., 1980), to the samples, the line-broadening effects were completely abolished, within experimental error, as indicated in Figure 2. These experiments establish that the observed change of the C-1 line width of the disaccharide is due to specific binding to the lectin. The data in Figure 2 correspond to a 26–82% saturation of the PNA binding sites.

Effect of Temperature on the Line Width of the C-1 Resonance of the Disaccharide in the Presence of PNA. The line broadening was found to increase with increasing temperature in the range from 10 to 30 °C as shown in Figure 3. In this temperature region, therefore, the disaccharide is in slow exchange on the ^{13}C NMR time scale between free and protein-bound environments, and the line-broadening effects are governed by the exchange rate $1/\tau_M$ (Dwek, 1973), which is equal to the dissociation rate constant of the disaccharide–PNA complex. Residence times, τ_M, and dissociation rate constants, k_{diss}, as well as dissociation equilibrium constants, K_D, of the sugar–protein complex, can therefore be determined

[2] D. R. Bundle, T. Iversen, and K. J. Neurohr, unpublished results.

[3] K. J. Neurohr, D. R. Bundle, N. M. Young, and H. H. Mantsch, unpublished results.

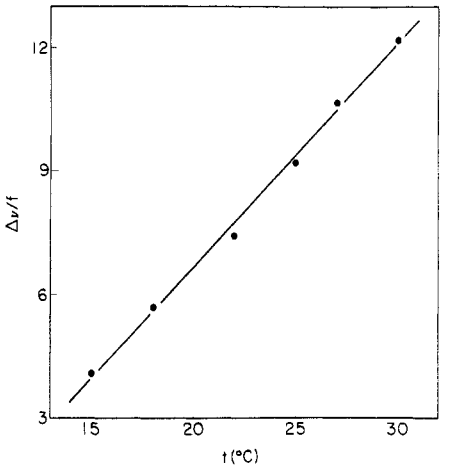

FIGURE 3: Temperature dependence of the C-1 line-width change of ^{13}C-labeled methyl β-D-lactoside in the presence of PNA in PBS at pH 7.2.

Table I: Temperature Dependence of the Lifetime in the Bound State (τ_M), Dissociation Rate Constant (k_{diss}), Equilibrium Constant (K_A), and Association Rate Constant (k_{assoc}) for the Binding of Methyl β-D-Lactoside to PNA[a]

T (°C)	$\tau_M \times 10^2$ (s)	k_{diss} (s^{-1})	$K_A \times 10^3$ (M^{-1})	$k_{assoc} \times 10^{-4}$ ($M^{-1}\ s^{-1}$)
15	7.7	13	3.6	4.8
18	5.6	18	2.7	4.8
22	4.4	23	1.8	4.1
25	3.5	29	1.4	4.0
27	2.9	33.9	1.1	3.7
30	2.3	43.5	0.9	3.8

[a] All data were obtained in PBS buffer, pH 7.2; estimated errors of τ_M, k_{diss}, and K_A are ±15%. k_{assoc} values were calculated from k_{diss} and K_A values obtained from UV difference spectroscopy; estimated errors ±20%.

from a study of the width of the C-1 resonance of the disaccharide in the presence of the lectin. The data were analyzed according to the equation

$$[S] = \frac{1}{\Delta\nu}\frac{n[P]}{\pi\tau_M} - K_D \qquad (1)$$

as described previously (Neurohr et al., 1981). Here [S] is the total disaccharide concentration, n[P] is the concentration of PNA binding sites, K_D is the dissociation equilibrium constant ($=1/K_A$), and τ_M is the residence time of the sugar on the protein ($=1/k_{diss}$). The number n of carbohydrate binding sites on the PNA tetramer was previously determined by equilibrium dialysis to be 4 (Neurohr et al., 1980). $\Delta\nu$ is the observed change in line width at half-height, defined as

$$\Delta\nu = \Delta\nu_{obsd} - \Delta\nu_{free} = \frac{1}{\pi T_{2\,obsd}} - \frac{1}{\pi T_{2\,free}}$$

A plot of [S] vs. $1/\Delta\nu$, according to eq 1, will yield K_D from the y intercept and the residence time τ_M from the slope. Figure 4 shows such a plot for the binding of the disaccharide to PNA at 30 °C. Similar plots are obtained at the other temperatures. Residence times, τ_M, dissociation rate constants, k_{diss}, and association equilibrium constants, K_A, for the binding of the disaccharide to PNA at different temperatures are summarized in Table I. Within experimental error, the same results were obtained at two different protein concentrations and with three different batches of freshly prepared PNA.

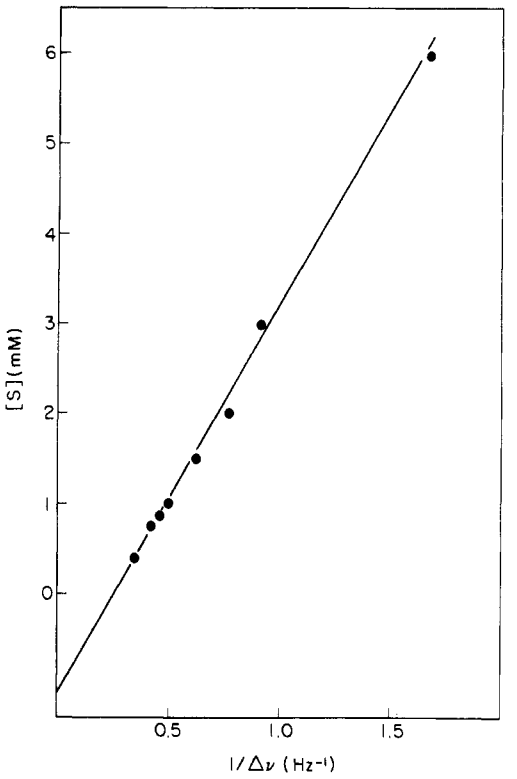

FIGURE 4: Plot of the total sugar concentration [S] vs. the reciprocal line broadening $1/\Delta\nu$ according to eq 1 for the binding of methyl β-D-lactoside to PNA at pH 7.2 and 30 °C.

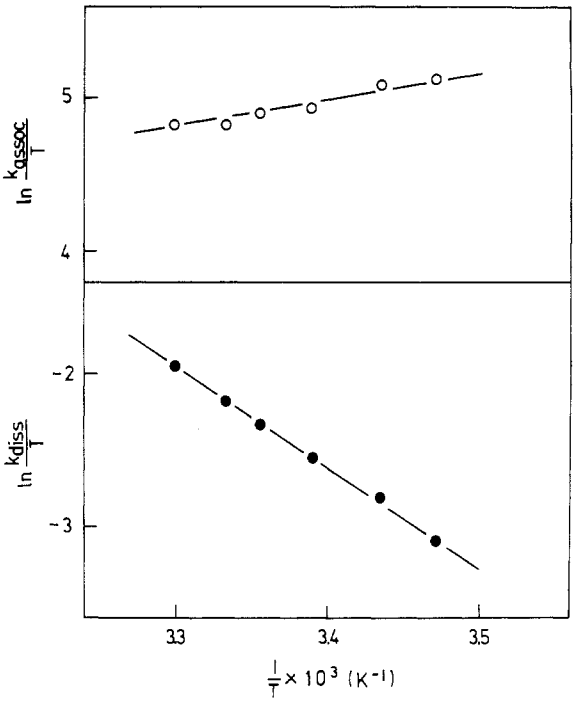

FIGURE 5: Plots of the association rate constant k_{assoc} (○) and the dissociation rate constant k_{diss} (●) for the binding of methyl β-D-lactoside to PNA at pH 7.2 according to eq 2.

Temperature Dependence of Association Constants. The association constants, K_A, for the binding of the disaccharide by PNA at different temperatures, as obtained from the y intercepts of plots according to eq 1 (which yield $K_D = 1/K_A$), are reported in Table I. The values of K_A are in excellent agreement with those determined independently by UV difference spectroscopy.[3] The value of the thermodynamic parameters $\Delta H°_{total}$ and $T\Delta S°_{total}$, as obtained from the present

Table II: Thermodynamic and Kinetic Data for the Binding of Methyl β-D-Lactoside to PNA[a]

parameter	value	
	kcal mol^{-1}	kJ mol^{-1}
ΔH°	−16.8	−70.3
$\Delta H^\ddagger_{assoc}$	−3.5	−14.6
$\Delta H^\ddagger_{diss}$	13.4	56.1
$T\Delta S^\circ$ [b]	−12.4	−51.9
$T\Delta S^\ddagger_{assoc}$ [b]	−14.6	−61.1
$T\Delta S^\ddagger_{diss}$ [b]	−2.0	−8.4
ΔG° [b]	−4.3	−18.0
$\Delta G^\ddagger_{assoc}$	11.1	46.4
$\Delta G^\ddagger_{diss}$	15.4	64.4

[a] $\Delta G^\circ = \Delta G^\ddagger_{assoc} - \Delta G^\ddagger_{diss}$, etc. All data were obtained in PBS at pH 7.2; estimated errors are ±1 kcal mol^{-1}, except for ΔG° (±0.1 kcal mol^{-1}), ΔH° (±0.4 kcal mol^{-1}), $T\Delta S^\ddagger_{diss}$ (±0.5 kcal mol^{-1}), and $\Delta G^\ddagger$ (±2 kcal mol^{-1}). [b] At 25 °C.

NMR measurements, are thus also in very good agreement with the UV derived values.

Temperature Dependence of Dissociation Rate Constants. Activation parameters were determined from linear plots of ln (k/T) vs. $1/T$ (Figure 5), according to the relationship

$$\ln \frac{k}{T} = -\frac{\Delta H^\ddagger}{R}\frac{1}{T} + \frac{\Delta S^\ddagger}{R} + \ln \frac{k'}{h} \qquad (2)$$

where k is the rate constant and k'/h is the ratio of the Boltzmann constant to the Planck constant. The activation parameters for dissociation of the disaccharide from the sugar–protein complex are summarized in Table II.

Discussion

The observation of ^{13}C resonances of specifically ^{13}C-labeled ligands is of considerable merit for the study of protein–ligand interactions. However, the potential of this method has not been extensively exploited for the study of protein–carbohydrate interactions, due in large part to the difficulty encountered in the synthesis of specifically ^{13}C-labeled carbohydrates, particularly oligosaccharides. The synthesis of methyl β-D-lactoside, specifically ^{13}C labeled at the C-1 position of the D-galactose residue, enabled us to extend previous ^{13}C NMR studies on PNA, which employed specifically ^{13}C-labeled methyl α- and β-D-galactopyranosides (Neurohr et al., 1981). The temperature dependence of the line-broadening effects established that the disaccharide was in slow exchange with the protein. The dissociation kinetics of the disaccharide with the lectin could therefore be determined from a study of the C-1 line width of the disaccharide in the presence of the lectin. Although rate constants can be obtained by chemical relaxation and stopped-flow measurements, these techniques usually involve the need for carbohydrates with bulky substituents as spectroscopic probes. The latter may perturb the system studied and significantly change the binding mechanism. The ^{13}C nucleus, on the other hand, represents a nonperturbing reporter group.

The excellent agreement of the association constants derived from NMR and UV spectroscopy indicates that both techniques monitor the same binding phenomenon. The NMR experiments described here and the UV experiments[3] were carried out under identical conditions of buffer, pH, ionic strength, and temperature. The protein concentration, however, is much larger in the NMR experiments [in the range of (6–9) × 10^{-5} M PNA tetramers] than in the UV experiments [in the range of (1.5–2) × 10^{-5} M PNA tetramers]. Both the NMR experiments and the UV difference experiments cover virtually the entire saturation range of the binding sites on PNA. The agreement between association constants and thermodynamic parameters determined by each technique shows that these parameters are not dependent on the protein concentration or the saturation range used in the experiments. The linearity of the plots in Figures 2 and 4 also rules out cooperativity effects in the PNA tetramer.

The thermodynamic parameters ΔH° and $T\Delta S^\circ$ obtained for methyl β-D-lactoside are quite different from those found for methyl β-D-galactopyranoside (Neurohr et al., 1981). The total enthalpy change for the disaccharide is considerably larger than that of the monosaccharide. However, this large increase in ΔH° is almost completely compensated by a corresponding more unfavorable entropy contribution of $T\Delta S^\circ = -12.4$ kcal mol^{-1}. The free energy change ΔG° on binding of the disaccharide is therefore similar to that for methyl β-D-galactopyranoside, resulting in similar association constants.

The dissociation rate constants found here for methyl β-D-lactoside are very similar in magnitude to those found for methyl α- and β-D-galactopyranosides. At 25 °C, k_{diss} is essentially the same for the disaccharide and the α anomer of methyl D-galactopyranoside. The dissociation rate constant of the disaccharide, however, shows a steeper temperature dependence than those found for the two methyl galactopyranosides. The activation parameters for dissociation in Table II indicate a larger activation enthalpy for the disaccharide of 13.4 kcal mol^{-1}, relative to 10.2 kcal mol^{-1} for methyl β-D-galactopyranoside. Interestingly, however, the free energy of activation for dissociation of $\Delta G^\ddagger_{diss} = 15.4$ kcal mol^{-1} is essentially the same for the di- and monosaccharide [Table II and Neurohr et al. (1981)].

The ^{13}C line-width measurements yield dissociation rate constants and equilibrium constants directly. Thus, association rate constants can also be calculated. In this case, association constants were determined independently by UV difference spectroscopy[3] and were found to be in excellent agreement with those derived from the NMR measurements. Association rate constants thus calculated are included in Table I. Interestingly, the association rate constant for the disaccharide at 25 °C of 4.0 × 10^4 M^{-1} s^{-1} is very similar to the association rates found for the α and β anomers of methyl D-galactopyranoside. At 25 °C, the decrease of the association rate constants in the series methyl α-D-galactopyranoside, 4.9 × 10^4 M^{-1} s^{-1}, methyl β-D-lactoside, 4.0 × 10^4 M^{-1} s^{-1}, and methyl β-D-galactopyranoside, 3.7 × 10^4 M^{-1} s^{-1}, parallels the decrease in their equilibrium constants 1.8 × 10^3, 1.3 × 10^3, and 1.0 × 10^3 M^{-1}, respectively (Neurohr et al., 1980, 1981). The association rate constants, as obtained from the dissociation rate constants and independently determined equilibrium constants, decrease with increasing temperature (Figure 5). This observation and the fact that the activation enthalpy for dissociation $\Delta H^\ddagger_{diss}$ is less than $|\Delta H^\circ|$ suggest that the binding of the disaccharide to the lectin is not a simple bimolecular process but rather involves a more complex mechanism. CD and UV measurements have indicated a ligand-induced conformational change in the protein upon sugar binding (Fish et al., 1978; Neurohr et al., 1980; N. M. Young, K. J. Neurohr, and R. E. Williams, unpublished experiments).

We have previously proposed a two-step binding mechanism for PNA–saccharide interactions (Neurohr et al., 1980). This is further substantiated by the present data. In the two-step binding mechanism, the disaccharide binds to the lectin by forming an initial complex at a rate near diffusional, followed by a conformational change to produce the final complex. Hence, the process can be described by the equation:

$$P + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (PS)_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (PS)_2 \quad (3)$$

$$K_1 = \frac{[(PS)_1]}{[P] + [S]} = \frac{k_1}{k_{-1}} \qquad K_2 = \frac{[(PS)_2]}{[(PS)_2]} = \frac{k_2}{k_{-2}}$$

With the assumption $k_2 \gg k_{-2}$, the lifetime τ_M of the disaccharide on the protein becomes

$$\tau_M = k_{diss}^{-1} = k_{-2}^{-1}$$

and the apparent association constant becomes

$$\frac{[(PS)_1] + [(PS)_2]}{[P][S]} = K_1(1 + K_2) \simeq K_1K_2$$

At 25 °C, $K_1K_2 = 1.4 \times 10^3$ M^{-1} and $k_{-2} = 29$ s^{-1}. K_1, the equilibrium constant for formation of the initial complex, will decrease with increasing temperature. The observation that the association rate constant k_{assoc}, now k_2K_1, decreases with increasing temperature can be accounted for in this model by the assumption that k_2 increases less rapidly with increasing temperature than K_1 decreases with increasing temperature. Moreover, the dissociation activation enthalpy $\Delta H^{\ddagger}_{diss}$ now represents the activation enthalpy for k_{-2}, which can be less than $|\Delta H°|$. The apparent activation entropy for association $T\Delta S^{\ddagger}_{assoc} = -14.6$ kcal mol^{-1} (Table II) is slightly larger than the corresponding value found for methyl β-D-galactopyranoside, $T\Delta S^{\ddagger}_{assoc} = -11.1$ kcal mol^{-1}.

An alternative pathway involving a slow rate-controlling conformational change in the protein prior to ligand binding is less likely since the plots of the rate constants and the van't Hoff plot of the equilibrium constant are linear with reciprocal temperature over the temperature range studied. A conformational change would be expected to show a temperature dependence.

So far, only con A has been the subject of detailed kinetic investigations (Brewer et al., 1972; Grimaldi & Sykes, 1975; Lewis et al., 1976; Clegg et al., 1977; Farina & Wilkins, 1980). Most kinetic studies have employed monosaccharide derivatives; only two studies were carried out with disaccharides. Thus, the binding kinetics of 4-methylumbelliferyl- and *p*-nitrophenyl-α-D-mannobioside to con A were investigated by stopped flow (van Landschoot et al., 1980a; Williams et al., 1978). The association rates were in the range of $(1–5) \times 10^4$ M^{-1} s^{-1}, and a considerable activation enthalpy was found for the binding reaction. These studies are difficult to compare with our NMR study on the binding kinetics of methyl β-D-lactoside to PNA due to the presence of two potential binding sites on the mannobiose disaccharide and the use of bulky reporter groups, which may significantly alter the binding mechanism.

Some conclusions about the nature of the binding site can be made on the basis of our NMR and UV studies of PNA. In the UV difference experiments on the binding of disaccharides by PNA, the difference spectra given by methyl β-D-lactoside were broadly similar to those generated by methyl β-D-galactopyranoside, indicating a similar perturbation of tyrosine residues in the PNA–monosaccharide and PNA–disaccharide complexes.

This observation suggested that the galactose residue of methyl β-D-lactoside binds at the same site as the methyl D-galactopyranosides. However, in the details of the difference spectra (shape and magnitude, particularly of the shoulder at 278 nm) there are ligand-specific effects. The association activation entropy found here for the disaccharide is similar to the corresponding value for both anomers of methyl D-galactopyranoside (Neurohr et al., 1981). These observations suggest that the conformational change induced in the protein upon sugar binding is similar for methyl α- and β-D-galactopyranoside and for methyl β-D-lactoside. For all three compounds, the overall binding mechanism can be described by eq 3. This would explain the similar association rates found for the mono- and disaccharide.

We interpret the thermodynamic data from the present ^{13}C NMR study to suggest there is an extended carbohydrate binding site on PNA. The total enthalpy change on binding for the disaccharide, $\Delta H° = -16.8$ kcal mol^{-1}, is considerably larger than that for methyl β-D-galactopyranoside, $\Delta H° = -10.2$ kcal mol^{-1}. This increase is too large to be attributed to a nonspecific hydrophobic binding contribution of the D-glucose residue as has been suggested for the binding of mannose oligosaccharides by con A (van Landschott et al., 1980b). Moreover, the inhibition experiments of Lotan et al. (1975) have indicated that aromatic aglycons of galactosides do not increase the binding of the parent sugars by PNA, excluding the existence of a hydrophobic binding region adjacent to the D-galactose site on this protein. Furthermore, con A shows opposite behavior in that para-substituted phenyl glycopyranosides of D-glucose and D-mannose are better inhibitors than the corresponding parent monosaccharides (Loontiens et al., 1973). It is probable that both pyranosyl moieties of methyl β-D-lactoside are involved in interactions with the residues of the PNA binding site, including hydrogen bonding as well as favorable nonpolar contacts. This subject will be discussed in more detail elsewhere.[3]

This study illustrates the thermodynamic differences that can be obscured by similar K_A values for related but distinct ligands. The assessment of relative inhibitor power, as in the hapten inhibition technique, merely reflects the ratio of binding constants for different ligands. In the absence of complementary thermodynamic and kinetic data, elaborate deductions of binding-site specificity and the details of the binding process from only the relative inhibitory behavior of related ligands would seem imprudent.

Acknowledgments

The authors are grateful to I.C.P. Smith for helpful suggestions and the use of the CXP-300 spectrometer.

References

Brewer, C. F., Marcus, D., Grollman, A. P., & Sternlicht, H. (1972) in *Lysozyme* (Osserman, A., Boychok, S., & Canfield, R., Eds.) pp 239–250, Academic Press, New York.

Clegg, R. M., Loontiens, F. G., & Jovin, T. M. (1977) *Biochemistry 16*, 167–175.

Dwek, R. A. (1973) *Nuclear Magnetic Resonance in Biochemistry*, pp 37–47, Clarendon Press, Oxford, United Kingdom.

Farina, R. D., & Wilkins, R. G. (1980) *Biochim. Biophys. Acta 631*, 428–438.

Fish, W. W., Hamlin, L. M., & Miller, R. L. (1978) *Arch. Biochem. Biophys. 190*, 693–698.

Goldstein, I. J., & Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem. 35*, 128–340.

Grimaldi, J. J., & Sykes, B. D. (1975) *J. Biol. Chem. 250*, 1618–1624.

Hughes, R. C., & Sharon, N. (1978) *Trends Biochem. Sci. (Pers. Ed.)* 3, N275–N278.

Lewis, S. D., Shafer, J. A., & Goldstein, I. J. (1976) *Arch. Biochem. Biophys. 172*, 689–695.

Loontiens, F. G., van Wauwe, J. P., de Gussen, R., & de Bruyne, C. K. (1973) *Carbohydr. Res. 30*, 51–62.

Lotan, R., Skutelsky, E., Danon, D., & Sharon, N. (1975) J. Biol. Chem. 250, 8518-8523.
Neurohr, K. J., Young, N. M., & Mantsch, H. H. (1980) J. Biol. Chem. 255, 9205-9209.
Neurohr, K. J., Young, N. M., Smith, I. C. P., & Mantsch, H. H. (1981) Biochemistry 20, 3499-3504.
Podder, S. K., Sorolia, A., & Bachhawat, B. K. (1978) FEBS Lett. 85, 313-316.
Serianni, A. S., Nunez, H. A., & Barker, R. (1979) Carbohydr. Res. 72, 71-78.
van Landschoot, A., Loontiens, F. G., Clegg, R. M., & Jovin, T. M. (1980a) Eur. J. Biochem. 103, 313-321.
van Landschoot, A., Loontiens, F. G., & de Bruyne, C. K. (1980b) Eur. J. Biochem. 103, 307-312.
Williams, T. J., Shafer, J. A., Goldstein, I. J., & Adamson, T. (1978) J. Biol. Chem. 253, 8538-8544.

Effects of Organic Acids on Tubulin Polymerization and Associated Guanosine 5'-Triphosphate Hydrolysis†

Ernest Hamel,* Anthony A. del Campo, Michael C. Lowe, Phyllis G. Waxman, and Chii M. Lin

ABSTRACT: We have examined the effects of a number of organic anions, which stabilize tubulin, on tubulin polymerization, associated GTP hydrolysis, and polymer morphology. While microtubule-associated proteins, as well as glycerol, induced formation of typical microtubules in a reaction coupled to GTP hydrolysis at an initial 1:1 stoichiometry, the organic anions had varying effects. Only 2-(*N*-morpholino)ethanesulfonate induced formation of structures with the morphology of microtubules. With glutamate, fructose 1,6-bisphosphate, piperazine-*N*-*N*'-bis(2-ethanesulfonate), glutarate, and glucose 1-phosphate, the predominant structures formed were sheets of parallel protofilaments rather than microtubules. Creatine phosphate induced the formation of clusters of rings. GTP hydrolysis was closely coupled to polymerization only with glutamate. With creatine phosphate, there was minimal GTP hydrolysis. With all other organic anions, GTP hydrolysis substantially exceeded polymerization at all time points, with the onset of hydrolysis significantly preceding the onset of turbidity development. Nevertheless, the rate of GTP hydrolysis was a sigmoidal function of tubulin concentration under all conditions examined, suggesting that tubulin–tubulin interactions are required for hydrolysis. All anion-induced reactions were temperature dependent and cold reversible, but only the creatine phosphate induced reaction was not inhibited by GDP, Ca^{2+}, or colchicine and did not require GTP.

We have observed that a number of organic anions exert impressive stabilizing effects on the colchicine binding activity of tubulin (Hamel & Lin, 1980, 1981b), and most of them also induce purified tubulin to polymerize in a temperature-dependent, cold-reversible reaction (Hamel & Lin, 1980, 1981a). We have characterized these properties most extensively with glutamate and have exploited them to develop a high-yield, large-scale purification of tubulin free of nucleosidediphosphate kinase and ATPase activities (Hamel & Lin, 1981a,b). In an effort to determine whether there was a common basis for the stabilizing and polymerizing properties of the various effective anions (carboxylates, phosphates, and sulfonates), we have now examined their effects on the morphology of the polymerized tubulin and on GTP hydrolysis associated with polymerization.

Materials and Methods

Materials

Monosodium glutamate was obtained from Grand Island, GTP, 2-(*N*-morpholino)ethanesulfonate (Mes),[1] and CP were from Sigma, Pipes was from Calibiochem, and [α-^{32}P]GTP was from Amersham. Organic anions were obtained as the sodium salts or free acids, and all stock solutions were adjusted to pH 6.6 with NaOH or HCl. GTP was repurified by triethylammonium bicarbonate gradient chromatography on DEAE-Sephadex A-25.

Proteins. Purified calf brain tubulin and heat-treated MAP's were prepared as described previously (Hamel & Lin, 1981a; Hamel et al., 1981). The tubulin used here was free of nucleosidediphosphate kinase and ATPase activities, electrophoretically homogeneous, freed of unbound nucleotide by gel filtration chromatography (Hamel & Lin, 1981a), and concentrated as described previously (Hamel et al., 1981a). It was stored at 30.5 mg/mL in 1.0 M glutamate in liquid nitrogen. Analysis of the bound nucleotide has demonstrated that the tubulin contains 1.9 mol of guanine nucleotide per mol of protein, half of which is GTP and half GDP. We have assumed that the GTP is bound in the nonexchangeable site and the GDP in the exchangeable site since a polymerization step followed by homogenization of the polymer in a GTP-free solution was a late step in the purification, and since further polymerization steps require at least stoichiometric amounts of GTP (Hamel & Lin, 1981ac).

The heat-treated MAP's (Fellous et al., 1977) had little ATPase or nucleosidediphosphate kinase activity (Hamel et al., 1981) as well as little GTPase activity (David-Pfeuty et al., 1979).

Methods

Tubulin polymerization was followed turbidimetrically (Gaskin et al., 1974) as described previously (Hamel & Lin,

† From the Laboratory of Medicinal Chemistry and Biology (E.H., P.G.W., and C.M.L.) and the Laboratory of Chemical Pharmacology (A.A.d.C. and M.C.L.), National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205. *Received June 12, 1981.*

[1] Abbreviations: MAP's, microtubule-associated proteins; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonate); Mes, 2-(*N*-morpholino)ethanesulfonate; CP, creatine phosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetate.