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Characterization of Concanavalin A Sugar Binding Site by ^{19}F Nuclear Magnetic Resonance†

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ABSTRACT: The binding of *N*-trifluoroacetyl-α- and β-D-glucosamine to dimeric and tetrameric concanavalin A was studied using ^{19}F nuclear magnetic resonance. Competition of methyl α-D-mannopyranoside with the trifluoro sugar indicates that the sugar binds to the carbohydrate binding site of concanavalin A. A great deal of rotational freedom of the trifluoroacetyl residue was observed for bound *N*-trifluoroacetyl-D-glucosamine anomers in complexes with two protein aggregation states. No chemical shift in the ^{19}F resonance of the probe sugar was observed upon its binding to concanavalin A. These results indicate that binding of the trifluoro sugar in the protein hydrophobic pocket previously identified by X-ray crystallographic analysis (Edelman, G. M., Cunningham, B. A., Reeke, G. N., Jr., Becker, J. W., Waxdal, M. J., and Wang, J. L. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 2580; Hardman, K. D., and Ainsworth, C. F. (1972), *Biochemistry 11,* 4910) is unlikely. The ^{19}F resonance is broadened noticeably when the sugar binds to concanavalin A containing Mn^{2+} in the transition metal site. This broadening was used to calculate the distance separating the fluorine nuclei and the Mn^{2+} ion. The mean distance between the fluorine nuclei of the bound sugar and the concanavalin A transition metal site was found to be 12 and 14 Å for the α and β anomer, respectively. This is in agreement with carbon magnetic resonance results independently obtained (Brewer, C. F., Sternlicht, H., Marcus, D. M., and Grollman, A. P. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 1007; Villafranca, J. J., and Viola, R. E. (1974), *Arch. Biochem. Biophys. 160,* 465). It strongly implies that the carbohydrate binding site in solution is not the hydrophobic pocket identified by X-ray crystallographic analysis.

Concanavalin A (Con A[1]) is a protein isolated from the jack bean (*Canavalia ensiformis*) (Sumner and Howell, 1936). It is one of a class of plant proteins called lectins. Lectins, including Con A, have the ability to bind to cell surfaces and for that reason have become widely used in exploring the structure and dynamics of cell surfaces (Sharon and Lis, 1972; Burger, 1973; Inbar *et al.*, 1971). Con A agglutinates embryonic tissue cells (Moscona, 1971) and various neoplastic cells in tissue culture (Inbar and Sachs, 1969). Adult and normal cells are not agglutinated and generally have a lower binding affinity for Con A. Upon binding, Con A often causes a change in the physiological functioning of its target cell (Cuatrecasas, 1973; Powell and Leon, 1970). Burger and Noonan (1970) have demonstrated that normal growth may be restored to virally transformed cells by treatment with trypsinized Con A.

Association of Con A with cell surfaces occurs primarily by virtue of its ability to bind certain carbohydrates on the cell surface (Wray and Waldborg, 1971; Allen *et al.*, 1972). Carbohydrates with the minimum structural requirements for binding to Con A contain residues with the D-arabinopyranoside configuration at the C-3, C-4, and C-6 positions (Goldstein *et al.*, 1965, 1973). There is one monosaccharide binding site per 25,500 molecular weight subunit of Con A (Yariv *et al.*, 1968).

For monosaccharide binding activity, one transition metal and one calcium ion must be bound to each Con A subunit (Kalb and Levitzki, 1968). The transition metal requirement is satisfied by several metal ions including diamagnetic zinc and paramagnetic manganese ions. The calcium requirement, however, is more specific. Only cadmium and strontium substitution yields an active protein (Shoham *et al.*, 1973).

Sedimentation equilibrium studies indicate Con A exists as a 110,000 molecular weight tetramer at pH 7.0 and higher and

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[1] Abbreviations used are: Con A, concanavalin A; NTFAGlcn, *N*-trifluoracetyl-D-glucosamine; NphGlcp, *p*-nitrophenyl α-D-glucopyranoside; αMeMan, methyl α-D-mannopyranoside; Mn-Con A, manganese enriched concanavalin A; Zn-Con A, zinc enriched concanavalin A; IphGalp, *O*-iodophenyl β-D-galactopyranoside; IphGlcp, *o*-iodophenyl β-D-glucopyranoside.

as a 55,000 molecular weight dimer at pH 5.5 and lower (Kalb and Lustig, 1968; McCubbin and Kay, 1971). These aggregates are composed of identical monomers of 25,500 molecular weight. The monomers are a single polypeptide or fragments of that peptide (Wang *et al.*, 1971).

The primary sequence and crystal structure of Con A have been reported (Edelman *et al.*, 1972; Hardman and Ainsworth, 1972). The distance between the sugar and transition metal binding sites, as determined from the X-ray analysis, was reported to be about 20 Å. Investigations of the solution structure of Con A using ^{13}C nuclear magnetic resonance (nmr) and the probe molecule methyl D-glucopyranoside indicate a much smaller separation of sugar and metal binding sites (Brewer *et al.*, 1973a,b; Villafranca and Viola, 1974). In a recent report based on X-ray analysis, Hardman and Ainsworth (1973) have suggested that Con A has two types of organic molecule binding sites. One site is a general hydrophobic molecule binding site and the other is a specific carbohydrate binding site.

An understanding of the biological activity of Con A must be predicated on a knowledge of the nature of sugar binding to Con A in solution. In view of the contradictory distances for the separation of the sugar and metal binding site determined from X-ray analysis and ^{13}C nmr measurements, it is of value to determine the separation in an independent study. We have measured the distance between the transition metal and sugar binding sites and examined several characteristics of the sugar binding site for the two aggregation states of the Con A. ^{19}F nmr and the probe molecule *N*-trifluoroacetylglucosamine (NTFAGlcn) were used in these studies.

Materials and Methods.

Chemicals. The NTFAGlcn used in these experiments was synthesized by the method of Wolfrom and Conigliaro (1969). A satisfactory elemental analysis, infrared spectrum, and decomposition point were found. Shellfish glycogen from Sigma was used after extensive dialysis against glass-distilled water to remove low molecular weight glycogen. Glassware was rinsed with acid and glass-distilled water prior to use. Stock solutions in these experiments were stored in Nalgene containers.

Con A Preparation. The protein was prepared from jack bean meal by the method of Agrawal and Goldstein (1967). The purified protein was homogeneous as judged by disc gel electrophoresis at pH 4.5 in 5, 7.5, and 10% polyacrylamide gels (Shepherd and Gurley, 1966). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Weber and Osborn, 1969) gave the band pattern characteristic of homogeneous Con A (Wang *et al.*, 1971; Abe *et al.*, 1971). The protein preparations were active as judged by their ability to precipitate shellfish glycogen (Poretz, 1968).

Zinc and manganese ion enriched Con A was prepared either by extensive dialysis of native Con A *vs.* the appropriate buffer solution made 2 mM in either manganese or zinc as well as calcium ions, or by demetallization (Kalb and Levitzki, 1968) and extensive dialysis *vs.* the appropriate buffer which was 1 mM in either zinc or manganese and calcium ions. Metal ion content of the solutions was monitored using atomic absorption spectroscopy. Less than 2% contamination by the opposing metal was found in both Zn–Con A and Mn–Con A. Protein concentrations were determined spectrophotometrically using extinction coefficients of $E_{280}(1\%) = 12.4$ at pH 5.1 and 13.7 at pH 7.0 (Yariv *et al.*, 1968).

Buffer solutions were 0.2 M in sodium chloride and 0.05 M in sodium acetate for measurements performed at pH 5.1. For measurements at pH 7.0, the solutions were 0.05 M in morpholinopropanesulfonic acid instead of sodium acetate. All solutions were prepared with deionized, glass-distilled water.

Spectrophotometric Titrations. The activity of Con A was quantitated by titrating Con A with NphGlcp (Hassing and Goldstein, 1970). Uv measurements were made with a Cary Model 15 spectrophotometer. The sample temperature was maintained at 22–25°. A change in the extinction coefficient of the NphGlcp results upon its binding to Con A. An isosbestic point at 290 nm was observed for the interaction. The absorbance difference (ΔA) due to sugar-chromophore in a solution alone and the sugar-chromophore in a Con A solution then may be expressed as

$$\Delta A = (\epsilon_S - \epsilon_{PS})[PS]$$

where ϵ_S and ϵ_{PS} represent molar absorbancies of free and bound PNPGlcp and [PS] is the molar concentration of the NphGlcp–Con A complex. The contribution to ΔA from a solution of the NphGlcp alone was calculated using an ϵ_S determined in each titration from the concentration dependence of the observed absorbance change in the region of large excess of NphGlcp. The extent of binding, θ, was determined noting that

$$\theta = \frac{[PS]}{[P_T]} = \frac{\Delta A}{\Delta A_\infty}$$

where $[P_T]$ represents the total concentration of NphGlcp binding sites and ΔA_∞ is the absorbance difference when all NphGlcp sites are occupied (Archer *et al.*, 1973). The binding parameters were evaluated using Scatchard's procedure (Scatchard, 1949).

Competition between αMeMan and NphGlcp for the saccharide binding sites on Con A was measured by titrating NphGlcp–Con A solutions with aliquots of αMeMan solutions.

Nmr Measurements. The ^{19}F nmr measurements were performed on a modified Varian DP-60 nmr spectrometer operating at a constant field of 14,100 G, or on a Varian HA-100 nmr spectrometer. The spectrometers were field-frequency locked using a capillary of trifluoroacetic anhydride in each experimental sample. Measurements were made at 28.5° in NTFAGlcn titrations of Con A. Various amounts of a mixture of α and β anomers of NTFAGlcn were added to the protein solutions. After completing each measurement of the ^{19}F spectrum of NTFAGlcn–Con A solutions, an excess of αMeMan, a potent inhibitor of Con A glycogen precipitating activity (Goldstein *et al.*, 1965), was added. The nmr spectrum was again observed. The differences in the nmr line widths between solutions with and solutions without αMeMan were taken as the line width contributions owing to the interaction of NTFAGlcn with Con A. Since contributions to line widths arising from field inhomogeneity are expected to be additive and equal for both cases, the above correction eliminates error in the transverse relaxation time T_2 resulting from magnetic field inhomogeneity.

In variable-temperature studies, temperatures from 0 to 30° were obtained by passing cooled compressed air over the samples in the magnet gap. Temperature variation during a spectrum accumulation was no more than ±1°. A single Con A–NTFAGlcn solution was used for each temperature variation. The homogeneity of the spectrometer was monitored in these experiments by observing the resonance of trifluoracetate which was present in the locking capillary.

Results

Spectrophotometric Titrations. The results of a typical spectrophotometric titration are shown in Figure 1. Analysis of the data by the method of Scatchard (1949) is shown in Figure 2. No cooperativity in sugar binding was observed in the prepara-

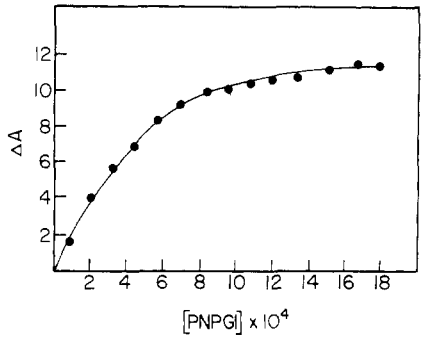

FIGURE 1: Results of a spectrophotometric titration of manganese-enriched Con A tetramers (pH 7.0) with NphGlcp. The initial concentration of Con A subunits is 0.475 mM. The change in absorbance ΔA is calculated as described in the text. Absorbance measurements were made using 1-mm path-length quartz cells in a double beam spectrophotometer. The reference cell contained the buffer which was used for the sample solution.

tions examined. Association constants and stoichiometry for the reaction of NphGlcp with various Con A's are summarized in Table I. The association constants are in reasonable agreement with those reported by Hassing and Goldstein (1970). The stoichiometry, one NphGlcp residue for each 25,500 molecular weight subunit, does not change over the pH range covered in this investigation. The association constants also are not sensitive to pH in the range investigated. This is in agreement with other studies of carbohydrate binding to Con A (Hassing and Goldstein, 1970; Yariv *et al.*, 1968).

The results of a αMeMan–NphGlcp competition experiment are shown in Figure 3. These experiments show that αMeMan displaces all NphGlcp from the Con A. An association constant for αMeMan binding to Con A was calculated to be $1.5 \pm 0.8 \times 10^4$, and was found to remain constant over a twofold range in protein concentration. This value is in good agreement with literature values (Hassing and Goldstein, 1970; Goldstein *et al.*, 1965).

Nmr Measurements. The effect of Con A on the ^{19}F nmr spectrum of NTFAGlcn is demonstrated in Figure 4. The resonance to low field corresponds to the NTFAGlcn α anomer and the high-field resonance to the β anomer (Millett and Raftery, 1972). The middle resonance in the first spectrum arises from a trifluoroacetate internal standard. When the sugar resonances were observed in the presence of Mn–Con A, a pronounced increase in the ^{19}F line width of both anomers was noted. No

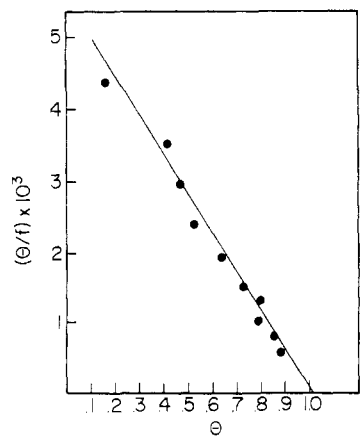

FIGURE 2: Analysis of a spectrophotometric titration of manganese-enriched Con A by the method described in the text. The data are shown in Figure 1. θ is the extent of NphGlcp binding and f is the concentration of free NphGlcp.

TABLE I: Parameters for Binding of NphGlcp to Con A.

Enriching Metal	pH	K_A[a] (M^{-1}) $\times 10^{-3}$	Binding Stoichiometry
Zn	5.1	5 ± 3[b]	1.05 ± 0.1[b]
Zn	7.0	6 ± 3	0.97 ± 0.05
Mn	5.1	5 ± 3	1.07 ± 0.1
Mn	7.0	4 ± 3	1.07 ± 0.1

[a] Association constant. [b] Listed errors are standard deviations.

chemical shift was observed in spectra of either anomer. The broadening could be eliminated by addition of αMeMan. When the spectrum of both anomers was observed in solutions containing 2 mM manganese but no Con A, a negligible increase in line width was observed. Manganese by itself, then, is not responsible for the observed broadening.

In contrast to the effect of Mn–Con A on the NTFAGlcn spectrum, Zn–Con A induced only moderate broadening. This slight broadening was also eliminated by the addition of αMeMan. Zn–Con A induced no chemical shift in the NTFAGlcn resonances. Figure 5 shows the results of a titration of Mn–Con A and Zn–Con A with the α anomer of NTFAGlcn.

Line broadening of the nmr spectrum of a small molecule due to a binding equilibrium with a macromolecule can be treated by the method of Swift and Connick (1962) when the exchange is between two sites and one site, the free sugar, is in excess over the other. These conditions hold in our experiment. Since no change in chemical shift was observed as a result of the equilibrium in either the Zn- or Mn–Con A, the Swift and Connick formulation is represented by

$$\frac{1}{T_{2P}} = \frac{f}{T_{2M} + \tau_M} \qquad (1)$$

where T_{2P} is defined by the relation

$$\frac{1}{T_{2P}} = \frac{1}{T_2} - \frac{1}{T_{2o}}$$

T_2 is the transverse relaxation time observed in a NTFAGlcn–Con A solution and, under conditions of no radiofrequency saturation, is related to the observed line width ω by $\omega = 1/\pi T_2$; T_{2o} is the transverse relaxation time corresponding to the same Con A sugar solution after addition of an excess of αMeMan. The lifetime of the sugar bound to Con A is τ_M. T_{2M} is the

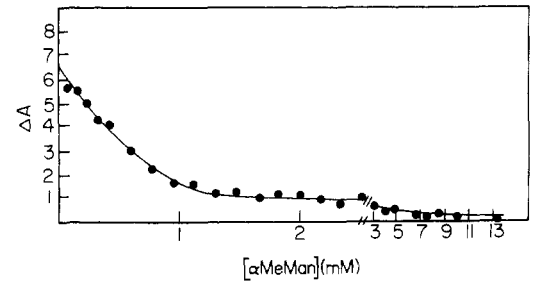

FIGURE 3: The competition of methyl α-D-mannopyranoside for the PNPGl binding sites on Con A. The solution's initial concentrations are 0.3 mM in Con A subunits, and 15.5 mM in NphGlcp. The titration was performed in the pH 7.0 buffer described in the text. ΔA was calculated as was described in the text and absorbance measurements were made as described in Figure 1.

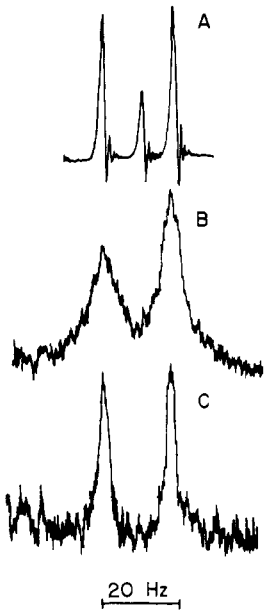

FIGURE 4: The effect of manganese-enriched Con A on the ^{19}F nmr spectra of α and β anomers of NTFAGlcn. Spectrum A is of a 0.1 M solution of NTFAGlcn at pH 5.1. Spectrum B is of a pH 5.1 solution 0.54 mM in Con A subunits and 3.5 mM in each anomer of NTFAGlcn. The nmr spectrum of the same Con A–NTFAGlcn solution made 0.1 M in methyl α-D-mannopyranoside is represented by C. The buffer used is described in the text.

transverse relaxation time characterizing the bound sugar and f is the fraction of sugar which is bound to Con A. As has been shown (Sykes *et al.*, 1970; Navon *et al.*, 1970; Lanir and Navon, 1971), f may be expressed in terms of the dissociation constant, K, the sugar anomer concentration, $[S_T]$, and the total Con A concentration, $[P_T]$, under the conditions required by eq 1. For the case in which the spectrum of one anomer of NTFAGlcn is observed in the presence of the other anomer, these authors' formulation is

$$[([P_T] - [PB])]/\omega_P = \pi(T_{2M} + \tau_M)(K + [S_T]) \quad (2)$$

The line width, ω_P, of the anomer whose spectrum is being observed was calculated as described in the Materials and Methods section. [PB] is the concentration of the complex of Con A with the other anomer. The plots obtained (Figure 6) from this relation were reasonably represented by a straight line despite uncertainty in [PB]. The plots were used to obtain estimates of K. These estimates were used to more accurately calculate ($[P_T] - PB$]) in eq 2. The titration data were replotted according to eq 2. In this way refined values of $(T_{2M} + \tau)$ and K were obtained. The values of $(T_{2M} + \tau_M)$ and K were then used with eq 1 to reproduce titration data such as those shown in Figure 5. The binding and relaxation parameters were adjusted until constant values of K were obtained throughout each titration. Using eq 1 in refining $(T_{2M} + \tau_M)$ and K caused these constants to change by no more than 15%. Curved lines in Figure 5 represent calculated values from refined data and demonstrate a close fit to experimental points. Binding parameters for the association of α and β anomers of NTFAGlcn with Mn-Con A at pH 5.1 and 7.0 are summarized in Table II. The same values for these parameters were obtained over a twofold range in both manganese concentration and protein concentration.

The line width increase resulting from the NTFAGlcn interaction with Zn-Con A was too slight to allow a similar analysis. The bound line width of NTFAGlcn in this case was estimated to be 5 ± 3 Hz for both anomers at pH 5.1 and 7.0.

The line width of both sugar anomers was found to decrease

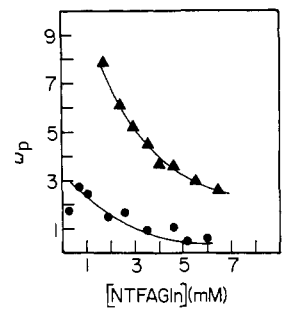

FIGURE 5: ^{19}F line width enhancement of varying concentrations of NTFAGlcn's α anomer in solutions of Con A at pH 7.0: (▲) manganese-enriched Con A solutions 0.47 mM in Con A subunits; (●) zinc-enriched Con A solutions 0.8 mM in Con A subunits. ω_p is the corrected line width described in the text. The points represent experimental values while the curves are calculated using eq 1 and 2. In the case of manganese-enriched Con A, constants from Table II are used in the calculation. In the case of zinc-enriched Con A, the binding constant is assumed to equal the binding constant for manganese-enriched Con A, and a bound line width of 8 Hz is used.

with increasing temperature over a temperature range from 0 to 30°. An activation energy for T_{2P} describing this dependence was calculated and found to be 2 ± 0.6 kcal. The NTFAGlcn interaction with Con A must, therefore, be in or near the fast exchange limit and τ_M is, therefore, small with respect to T_{2M} and may be disregarded in eq 1 and 2. The analysis of NTFAGlcn titration results, then, yields values of T_{2M} directly. The line width for both sugar anomers in Mn-Con A solutions were the same when the spectra were measured either at 94.1 or 56.4 MHz.

Discussion

A quantitative knowledge of the saccharide binding activity of Con A is required to fully interpret our nmr titrations using the analysis described in the Results section. A thorough knowledge of the activity must include definition of the stoichiometry and cooperativity, if any is displayed. A characterization of the binding of NphGlcp to Con A enables one to analyze the activity of each Con A subunit regardless of its aggregation state.

The binding of NphGlcp to all Con A preparations examined (Table I) is saturable (Figure 1). We find one NphGlcp binding site per 25,500 molecular weight subunit of Con A and no secondary binding sites (Figure 2). Within the limits of our analysis (Figure 2), the subunits show no cooperativity in binding sugar regardless of their aggregation state. αMeMan quantitatively displaces NphGlcp from Con A (Figure 3). The asso-

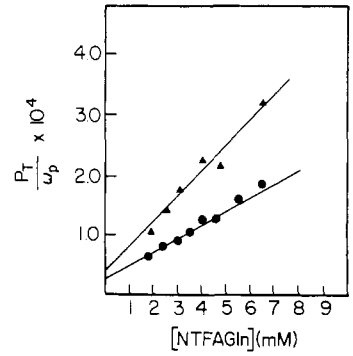

FIGURE 6: Initial analysis of data for NTFAGlcn interaction with manganese enriched Con A at pH 7.0: (●) the sugar α anomer; and (▲) the sugar β anomer. P_T is the total concentration of Con A subunits and ω_P is the corrected line width described in the text. The plot is made according to eq 2 in the text.

TABLE II: Parameters for the Binding of NTFAGlcn to Mn–Con A.

Con A Aggregation State[a]	NTFAGlcn Anomer	K_A[b] (M^{-1}) × 10^{-2}	T_{2M} (sec) × 10^2	ω_p	^{19}F–Mn Distance
Dimer	α	6.8 ± 3[c]	1.3 ± 0.3[c]	74 ± 19[c]	12.2 ± 0.5[c,d]
	β	6.4 ± 3	2.9 ± 0.8	35 ± 9	14.0 ± 0.5
Tetramer	α	8.2 ± 2	1.4 ± 0.1	74 ± 7	12.2 ± 0.2
	β	7.8 ± 3	2.8 ± 0.8	36 ± 11	14.0 ± 0.6

[a] Obtained by varying the pH of the experimental solutions (Kalb and Lustig, 1968). [b] Association constant. [c] Listed uncertainties are standard deviations. [d] Errors in τ_c used in eq 3 may lead to larger errors than the standard deviations.

ciation constant for the αMeMan association with Con A, measured using this displacement reaction, agrees with the value measured directly for the association reaction (So and Goldstein, 1968). These observations and the finding that the equilibrium constant for the displacement reaction remains constant over a range of Con A concentrations indicate NphGlcp and αMeMan are binding to the same site or closely linked sites on Con A (Edsall and Wyman, 1958). We have further shown that αMeMan displaces NTFAGlcn from Con A (Figure 4). This indicates that all three monosaccharides probably bind to the same site on Con A. Since αMeMan inhibits binding of a variety of polysaccharides (Poretz, 1968), this site most certainly is responsible for part or all of the polysaccharide binding activity shown by the lectin. Finally, since the binding parameters for NphGlcp binding to Mn–Con A and to Zn–Con A are the same (Table I), the binding of NTFAGlcn and αMeMan to both Mn–Con A and Zn–Con A also is certainly very similar.

Nmr Results. In addition to its binding properties, NTFAGlcn makes a good binding site probe for Con A because the trifluoromethyl group is particularly well suited for reporting its magnetic environment (Millett and Raftery, 1972; Buckingham *et al.*, 1960; Pople *et al.*, 1959).

The absence of an observed chemical shift of the resonances of either anomer when interacting with Con A at pH 7.0 or 5.1 is surprising if the sugar is binding in the pocket suggested by crystallographic results (Edelman *et al.*, 1972; Hardman and Ainsworth, 1972). The proposed binding pocket is 18 Å deep and 6 Å by 3.5–7.5 Å in cross section. Hydrophobic residues including tryptophan, tyrosine, and phenylalanine line this pocket. In view of the limited cross-sectional area of the pocket relative to NTFAGlcn dimensions, a shift resulting from magnetic anisotropy of aromatic side chains or van der Waals interactions with any side chains lining the cavity might be expected. Our results indicate that the NTFAGlcn is binding in a manner in which these interactions do not occur with sugar trifluoromethyl group.

The distance separating manganese ion and NTFAGlcn bound to Con A can be calculated from the enhancement of the nmr transverse relaxation rates of the bound NTFAGlcn residues caused by manganese. Since uniform binding constants and T_{2M}'s were obtained for varying protein and manganese concentrations, viscosity effects must contribute negligibly to T_{2M}, and the binding of manganese to secondary sites close to the sugar site on Con A is unlikely. Brewer and coworkers (Brewer *et al.*, 1973a,b) also have concluded that there is no secondary manganese binding to Con A. As indicated in the Results section, exchange does not contribute significantly to the observed relaxation rates. The relaxation enhancement produced by manganese, then, can be obtained by correcting the T_{2M} for a Mn–Con A–sugar complex by the T_{2M} for the corresponding Zn–Con A–sugar complex. This approach assumes that saccharide binding to Zn–Con A and Mn–Con A is the same. This is strongly suggested by our measurements of NphGlcp binding to Mn–Con A and Zn–Con A (Table I). Shoham *et al.* (1973) have also presented data concerning the binding of methyl α-D-glucopyranoside to both forms of Con A and suggest that it is equivalent.

The relationship describing the effect of a paramagnetic center on the transverse relaxation rate of high resolution nuclei (eq 3) is well known (Connick and Fiat, 1966; Fung, 1973).

$$(1/T_{2M}) = \mathrm{DD}[f(\tau_c)/r^6] + Z \qquad (3)$$

The second term on the right-hand side is disregarded since it involves the binding of manganese ions to the fluorines of NTFAGlcn. This most certainly is not the case. DD is the term describing the magnitude of the dipolar coupling of the ^{19}F nuclei and the manganese electrons and is given explicitly by $[s(s+1)\gamma_I^2\beta^2/15]$, where s is the electronic spin quantum number of the manganese ion, γ_I is the nuclear magnetogyric ratio, g is the Landê g factor, and β is the Bohr magneton. The distance separating the ^{19}F nuclei from the paramagnetic center is r and $f(\tau_c)$ is a function of the correlation time describing the electron ^{19}F interaction.

To measure distances, the correlation time and nature of the correlation function must be determined. The dipolar correlation time τ_c in Mn–Con A–sugar complexes is a composite of a fluorine–manganese rotational correlation time τ_R, the lifetime of the NTFAGlcn–Con A complex τ_M, and the electron spin

$$1/\tau_c = 1/\tau_R + 1/\tau_M + 1/\tau_S$$

relaxation time τ_S. The lifetime τ_M of the sugar–manganese complex is probably 10^{-2}–10^{-5} sec (Brewer *et al.*, 1973a; Dwek, 1973). It is large compared with the τ_c determined for the Zn–Con A–NTFAGlcn complexes. Since τ_S does not apply to this complex, τ_M must make an insignificant contribution to τ_c. The electron spin relaxation time τ_S is also large with respect to τ_c and, therefore, contributes negligibly (Koenig *et al.*, 1973). The rotational correlation time τ_R must dominate the τ_c for the Con A–sugar complexes. The correlation time may be estimated from the Zn–Con A effect on the transverse relaxation rate of ^{19}F NTFAGlcn. This estimation assumes that intramolecular nuclear interactions are responsible for the relaxation of ^{19}F nuclei of NTFAGlcn bound to Zn–Con A. Intramolecular mechanisms for the relaxation of methyl groups on small molecules bound to diamagnetic macromolecules are believed to be the primary type of relaxation mechanisms (Marshall, 1972; Anderson *et al.*, 1970; Lanir and Navon, 1971). This is quite resonable in view of the close proximity of neighboring nuclei in methyl groups, and the inverse r^6 dependence of dipolar interactions. Most likely these dipolar mechanisms are dominant for the fluorine nuclei of NTFAGlcn bound to Con A. Certainly the lack of observed chemical shifts, which

would be expected if the trifluoromethyl group were influenced by Con A residues when bound to Con A, supports the suggestion that relaxation is by intramolecular processes.

The relation describing the intramolecular relaxation of ^{19}F nuclei in trifluoromethyl groups bound to diamagnetic macromolecules (eq 4) has been given by Werbelow and Marshall

$$\frac{1}{T_2} = \frac{3\gamma_I{}^4\hbar^2}{10r^6}\left[3\tau_c + \frac{2\tau_c}{1 + 4\omega^2\tau_c{}^2} + \frac{5\tau_c}{1 + \omega^2\tau_c{}^2}\right] \quad (4)$$

(1973a). The correlation time for the motion of the ^{19}F nuclei is τ_c and h is Planck's constant divided by 2π. This relation assumes that relaxation is exponential as would be the case if the ^{19}F nuclear motion was represented by the motion of a point on a randomly reorienting sphere. This would be a good model if the NTFAGlcn trifluoromethyl groups were rigidly bound to Con A. Line widths from our Zn-Con A titrations, however, are too small to be accounted for by assuming no internal rotation of the trifluoromethyl group. The correlation times used to calculate expected line widths in the absence of internal rotation are those describing the motion of the protein as a whole, $3\text{-}8 \times 10^{-8}$ sec. These values were determined by nmr dispersion and ^{13}C nmr measurements on Con A (Brewer *et al.*, 1973; Koenig *et al.*, 1973). Free rotation of the trifluoromethyl group or the trifluoroacetate group, therefore, must occur. This is expected to change the form of the correlation function associated with ^{19}F relaxation.

Recent reports by Werbelow and Marshall (1973a) and Levine *et al.* (1973) allow an estimation of both the severity of using the approximation of exponential relaxation rates in the presence of internal rotation and the effect on observed relaxation rates of allowing several axes of internal rotation between the observed nucleus and the macromolecule to which it is attached. The exponential approximation is most severe when rapid axial rotation of a methyl group occurs with a much slower random reorientation of the single rotational axis. Werbelow and Marshall (1973b), however, have shown that nonexponential relaxation is not likely to be observed for freely rotating CF_3 groups. Solving explicitly for the case of rapid rotation of a methyl group with a much slower random reorientation of its rotational axis, these authors have shown the maximum effect of such a rotation woud be to increase the observed T_2 by a factor of 4 over its value in the absence of internal rotation (Werbelow and Marshall, 1973a). The ω_P's determined from our Zn-Con A titrations are too small to be accounted for by assuming only one axis of internal rotation. The trifluoroacetyl group, as well as its trifluoromethyl portion, therefore must be able to rotate.

Levine and coworkers (Levine *et al.*, 1973) have found that the relaxation of magnetic nuclei and side chains of macromolecules rapidly becomes independent of the macromolecular motion as the number of rotational axes between the magnetic nucleus and the macromolecule increases. This explains our observation that T_{2M}'s determined from Zn-Con A (Results section) and Mn-Con A measurements (Table II) are the same for dimeric Con A and tetrameric Con A. Since the correlation time of the protein motion is expected to change, and yet is not detected in the nmr experiment, more than one axis of rotation must be present. This observation provides additional evidence for the rotation of the entire trifluoroacetyl group when bound to Con A.

With two axes of rotation, the relaxation of the ^{19}F nuclei becomes more accurately represented as an exponential process (Levine *et al.*, 1973). An error of no more than a factor of 4 in the correlation times calculated using eq 4 is expected as a result of approximating the relation as exponential. A correlation time of 1.2×10^{-9} sec was determined using relaxation times for NTFAGlcn bound to Zn-Con A and fluorine-fluorine distances determined by X-ray crystallography (Chang *et al.*, 1970).

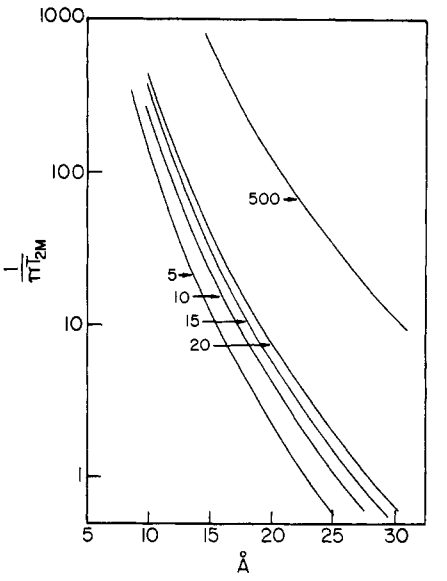

FIGURE 7: The dependence of $1/\pi T_{2M}$ for a ^{19}F nucleus on its separation from a manganese ion. The different lines represent different values of the correlation time (τ_c) for the interaction. The values are expressed in seconds and are multiplied by 10^{10}. T_{2M} is described in the text. $1/\pi T_{2M}$ equals the line width of fluorine on the bound NTFAGlcn.

The determined correlation time is consistent with our observation that Con A shows no field dependence in its enhancement of the NTFAGlcn nmr line widths. Correlation times either slower than 6×10^{-9} sec or faster than 1.5×10^{-9} sec would exhibit this behavior (Navon and Lanir, 1972; Connick and Fiat, 1966). The slower limit is too slow to be consistent with our Zn-Con A titration results, but the faster limit agrees quite well with the correlation time just determined. Higher field measurements of T_1 and T_2 would be expected to show a field dependence (Werbelow and Marshall, 1973a; Navon and Lanir, 1972). These measurements would be desirable in confirming our assignment of τ_c, but require instrumentation currently unavailable to the authors. Our measurements at 56.4 and 94 MHz do put a reasonable upper limit on the correlation time of 1.2×10^{-9} sec. Higher field measurements might show dependence and any shorter correlation time so determined would place the trifluoromethyl group closer to the manganese ion.

Figure 7 shows the dependence of the observed relaxation rate, as described by eq 3, on the separation of the manganese ion and the ^{19}F nuclei for several values of the correlation time. The correlation times used are consistent with values obtained from NTFAGlcn binding studies with Zn-Con A. Using an effective correlation time of 1.2×10^{-9} sec, eq 3 was used to calculate the average separation between the manganese ion bound to Con A and the ^{19}F nuclei in NTFAGlcn also bound to Con A. The distances and binding constants characterizing the binding of the α and β anomer to dimeric (pH 5.1) and tetrameric (pH 7.0) Con A are summarized in Table II. The uncertainty in the absolute distances resulting from the correlation time is expected to be no more than ±10-15% and is expected to be the limiting source of uncertainty. The uncertainty in absolute distances in Table II must include any relaxation

effects resulting from fluctuations in the Mn–F distances. Such fluctuations could arise as a result of the mobility of the trifluoroacetate moiety in bound NTFAGlcn. The distances are viewed as a type of average of the positions available to the trifluoroacetate group. Relative distances, however, are not subject to this uncertainty. Reliability in this sense is represented in Table II. The values of the binding constants are in good agreement with the binding constant of 600–700 M^{-1} for *N*-acetyl-D-glucosamine estimated from dextran precipitation studies with αMeMan and *N*-acetyl-D-glucosamine (So and Goldstein, 1968; Goldstein *et al.*, 1965).

A model was constructed placing NTFAGlcn carbons at distances from the manganese ion determined by ^{13}C nmr measurements (Brewer *et al.*, 1973a,b). These authors found the average sugar carbon–manganese distance to be about 10 Å. Average ^{19}F–manganese distances of 12 and 14 Å we measured are in agreement with this model. Variation in the α and β NTFAGlcn anomer fluorine–manganese distances probably reflect a slight change in preferred conformation of NTFAGlcn as a result of the change in stereochemistry about the C-1 sugar carbon.

Temperature-dependent measurements reported by Brewer *et al.* (1973b) indicate that methyl α- and β-D-glucopyranoside are bound to Con A in a manner characterized by an intermediate exchange condition, In contrast, we observed rapid exchange for both the anomers of NTFAGlcn. The difference in the kinetics of the interaction most likely arises from small differences in the binding of the sugars as a result of the altered sugar structure. The addition of a methyl group at the C-1 position of a hexopyranoside substrate is known to enhance the substrate binding ability (Poretz and Goldstein, 1970). Perhaps the interaction of this residue with Con A is responsible for the kinetic difference between methyl D-glucopyranoside and NTFAGlcn binding.

Our conclusions based on ^{19}F mnr and those reached by Brewer *et al.* (1973a,b) and Villafranca and Viola (1974) are in reasonable agreement and place the sugar binding site about 10 Å from the metal binding site in the solution structure of Con A. These findings do not agree with X-ray crystallographic analysis which suggests the sugar residues bind in a hydrophobic pocket 20 Å away from the manganese binding site (Edelman *et al.*, 1972; Hardman and Ainsworth, 1972). These authors realized, however, that the IphGlcp residue used as a substrate analog to locate the protein was bound to crystalline Con A in an anomalous manner. The specificity of Con A requires a definite configuration of the C-3, C-4, C-6 end of the sugar (Goldstein *et al.*, 1965), but the crystallographic determination suggests that this portion of the sugar is directed out of the proposed binding pocket.

Assuming the crystal structure of Con A accurately represents the solution structure, our distance measurements allow the NTFAGlcn residue to be placed either extremely deep in the hydrophobic pocket or at some other location not involving the pocket at all. The absence of a chemical shift of bound NTFAGlcn fluorine magnetic resonances, and the observed mobility which is retained by the trifluoroacetate residue on NTFAGlcn bound to Con A, indicate that this sugar is not binding in the hydrophobic pocket. A more reasonable position for the bound sugar is on or near the protein surface. Our investigation and those of Brewer and coworkers (1973a,b) and Villafranca and Viola (1974) further indicate that the sugar is bound at a position about 10 Å from the metal binding site of the protein.

Recently Hardman and Ainsworth (1973) have provided X-ray crystallographic evidence suggesting that the pocket initially characterized as a sugar binding site may, in reality, be a general binding site for hydrophobic molecules. These authors suggested that the true carbohydrate binding site might be on the surface of the protein 10 Å from the manganese binding site. This suggestion is in agreement with our ^{19}F nmr and previously reported ^{13}C nmr measurements performed on Con A in solution.

Several recent observations are easily reconciled with the Con A sugar binding site being distinct from the hydrophobic pocket. Goldstein *et al.* (1973) provided evidence that Con A interacts with internal (1–2) linked D-mannopyranosyl residues of polysaccharides. If binding were to occur in the hydrophobic pocket, a great deal of steric hindrance would have to be overcome which might not be encountered if binding were at the protein surface. Noonan and Burger (1973) have reported that binding of Con A to cell surfaces is not totally dependent on carbohydrate specificity since αMeMan competes for only about 50% of the Con A bound to cell membranes. This suggests that Con A may have at least two means of interacting with the cell surface, only one of which is carbohydrate dependent. The second method of interaction might involve the hydrophobic pocket. Hardman and Ainsworth (1973) observed that IphGalp binds to crystalline Con A in the same manner as IphGlcp but unlike IphGlcp it will not inhibit dextran precipitation activity. This suggests the carbohydrate binding site is not the site at which both IphGalp and IphGlcp bind and, therefore, is not the hydrophobic pocket. Finally, we cannot rule out the possibility that Con A may exhibit a degree of conformational mobility (Barber and Carver, 1973; G. M. Alter and J. A. Magnuson, unpublished results) and possess a solution structure which is substantially different from its crystalline structure. Future work in this area might employ other metal ion binding sites in Con A. With X-ray localization of other sites, such as one for a second paramagnetic metal, the nmr studies could be extended considerably.

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