The binding of p-nitrophenyl 2-0-α-D-mannopyranosyl-a-D-mannopyranoside (M2) to concanavalin A was studied by equilibrium dialysis and uv difference spectroscopy. Concanavalin A, purified by conventional methods, was shown to behave heterogeneously in its interactions with M2 and Sephadex G-75. Elution of concanavalin A bound to Sephadex G-75 by 19 mm D-glucose gives a protein composed predominantly of "nicked" or fragmented polypeptide chains. As second elution with 100 mm D-glucose gives concanavalin A composed entirely of intact polypeptide chains. Both elution with 100 mm D-glucose gives concanavalin A more than the monosaccharide p-nitrophenyl α-D-mannopyranoside (M1). This increased affinity for the chromogenic disaccharide over its monosaccharide counterpart strongly suggests that concanavalin A interacts simultaneously with groups on both mannopyranosyl residues of M2.

Concanavalin A (con A)1 is a carbohydrate-binding protein.

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1 The abbreviations used are: con A, concanavalin A; M2, p-nitrophenyl 2-0-α-D-mannopyranosyl-α-D-mannopyranoside; SDS, sodium dodecyl sulfate; M1, p-nitrophenyl α-D-mannopyranoside.

**Materials and Methods**

RESULTS AND DISCUSSION

The formation of a complex between con A and a carbohydrate ligand as represented by Equation 1 is a symmetrical reaction, i.e.

\[
P + L \rightarrow P + L
\]  

(1)

2 Portions of this paper (including "Materials and Methods," Figures 1 to 5, and Refs. 14 to 19) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, Request Document No. 78M-77, cite author(s), and include a check or money order for $1.00 per set of photocopies.
interchanging the concentrations of P and L should not affect the amount of PL formed. Such behavior is seen in the interaction between con A and p-nitrophenyl α-D-mannopyranoside (M2). Interestingly, this behavior in the interaction of M2 with con A is not observed at pH 5 or 7 either with a commercial source of the lectin or with the protein as prepared by affinity chromatography on Sephadex G-50 (6). Fig. 1 displays the asymmetric response seen in difference spectra for formation of a con A-M2 complex when solutions of con A and M2 are mixed.

It is evident that the apparent amount of complex formed as reflected by the magnitude of the difference spectra, depends on which component is in excess. An absorptivity change (317 nm) of \(-1.6 \times 10^3\) cm\(^{-1}\) M\(^{-1}\) (based on the concentration of con A protomeric units) was determined from the limiting change of absorbance when con A was titrated with excess M2, whereas in absorptivity change of \(-3.2 \times 10^3\) cm\(^{-1}\) M\(^{-1}\) (based on the concentration of M2) was observed when M2 was titrated with excess con A. The results of an equilibrium dialysis experiment depicted by the Scatchard plot in Fig. 2 show that the observed difference in absorptivities is not due to a 2:1 stoichiometry for a M2-con A complex.

The complexity of the binding reaction as reflected in Fig. 1 and the nonlinear Scatchard plot in Fig. 2 might be attributed to: 1) impurities in the M2 preparation, 2) certain components in the con A sample having different affinities and changes in molar absorptivity on binding M2, or 3) the affinity of con A binding sites or the molar absorptivity of a protomer-carbohydrate complex (or both) being dependent on the occupancy of the adjacent site on dimeric con A molecules.

Studies of the structure and purity of M2 indicated that the complexity of the binding reaction cannot be explained in terms of an impurity in M2 (see “Materials and Methods”). Since con A as isolated conventionally exists as a mixture of intact and nicked protomer subunits (5), a method was developed to purify large quantities of these species so that they might be examined as a source of the heterogeneous-like behavior seen in the Scatchard plot and ultraviolet difference spectra. Pure “intact” con A, enriched “nicked” preparations, as well as a reconstituted mixture of these two forms, prepared by the described method, yield spectra identical with that obtained for a solution of 100 μM con A and 40 μM M2 (Fig. 1) regardless of which species was in excess. This result indicates that simple nicking of the con A is not the cause of the described heterogeneity with respect to M2. A comparison of spectra also was obtained with M2 and intact, nicked, or an equal mixture of the two forms of con A, wherein the concentrations of the ligand and protein were equal to those required for 50% complex formation in the case of M2 and intact con A. The results of this study yielded three spectra of equal intensity, implying that all three preparations had the same concentration of complex and thus the same association constant.

Scatchard plots for intact con A are shown in Figs. 3 and 4. The data for the plot in Fig. 3 were obtained from equilibrium dialysis studies. The dependence of the concentration of bound ligand on the concentration of free ligand for the plot in Fig. 4 was determined spectrophotometrically using a value of \(-3.2 \times 10^3\) cm\(^{-1}\) M\(^{-1}\) for the change in molar absorptivity on formation of the complex. This value was obtained from the limiting change of absorbance when M2 was titrated with excess intact con A. The Scatchard plots in Figs. 3 and 4 indicate that the intact con A behaves homogeneously in its interactions with M2 in accord with Reaction 1. These data yielded (see “Materials and Methods”) the weighted average values (12) of 1.9 (±0.2) \(\times 10^5\) M\(^{-1}\) for K, and 0.93 (±0.02) M-binding sites per protomeric unit.

Although the con A fractions which were homogeneous with respect to binding M2 are easily displaced from the column by d-glucose, roughly 50% of the total con A was not eluted at 4°C or 25°C by 1.0 M d-glucose, 1.0 M methyl α-D-mannopyranoside, 1.0 M sodium thiocyanate, 0.2 M acetic acid, or 8 M urea either in the presence or absence of 50 mM CaCl\(_2\) and MnCl\(_2\). Rechromatography of the intact con A results in complete recovery of this material, indicating that the failure to obtain a quantitative recovery with commercially prepared con A is not an artifact of the procedure.

The data in Figs. 2, 3, and 4 and Equation 3 were analyzed to obtain an estimate (see “Materials and Methods”) of the amount of material (54%) in conventionally prepared con A which has a lower affinity for M2 as well as an estimate of the affinity \(K = 2.4 (±0.9) \times 10^4\) M\(^{-1}\) for M2. The estimated amount of this material is in reasonable agreement with the amount of material observed to be retained on the Sephadex G-75 column when the commercially prepared con A used for the study in Fig. 2 was subjected to chromatography on a Sephadex G-75 column. This result is consistent with the hypothesis that the material remaining on the column is a component in the original mixture which has a lower affinity for M2 than the eluted material.

Similar losses of con A on Sephadex G-75 columns have been reported by Cunningham et al. (13), who lost 30 to 50% of their con A in a purification of this protein on Sephadex G-75 at pH 7.2. Since binding studies were not performed with disaccharides, Cunningham et al. (13) did not observe altered carbohydrate interactions with the eluted material. Cunningham et al. (13) did show, however, that the amino acid composition of con A did not change upon chromatography. This result together with our observation that the combined eluted material gives patterns (on SDS electrophoresis) similar to those obtained with conventionally prepared con A is consistent with the view that the structural differences between the material eluted and retained on the column are small. Our observation that solutions of conventionally prepared con A, but not intact con A, develop precipitates on standing in low ion strength buffers near neutrality strongly suggests the possibility that the low affinity form of con A precipitates on the Sephadex column during this purification procedure, which is carried out at pH 7.2 and low ionic strength. Furthermore, if the chromatographic procedure is replaced by an incubation in 0.02 M Tris buffer, pH 7.2, at 4°C, 36 h, followed by the routine dialysis against pH 5, 0.5 M acetate buffer containing 0.1 mM Ca\(^{2+}\) and Mn\(^{2+}\), the resulting material, after removal of the insoluble material, is markedly enriched in components which give a homogeneous response toward M2. Finally, when the above chromatographic procedure is carried out at higher ionic strength (1/2 0.5, with NaCl) to prevent precipitation of the con A, 92% of the con A is recovered.

In an effort to probe further the relationship between the forms of con A which interact differently with M2, the effect of Ca\(^{2+}\) and Mn\(^{2+}\) on this interaction was studied, since the affinity of con A for its ligands has been correlated with its metal ion content (7–9). The presence of 1 mM Ca\(^{2+}\) and Mn\(^{2+}\) was found to be sufficient to cause the change in absorbance produced on mixing excess M2 with conventionally prepared con A to become equal to the change in absorbance produced by mixing excess M2 with intact con A. Furthermore, in the presence of 1 mM Ca\(^{2+}\) and Mn\(^{2+}\), the difference spectrum is no longer altered when the concentrations of M2 and conventionally prepared con A are interchanged. However, either...
Ca\(^{2+}\) or Mn\(^{2+}\) added alone are not effective in causing conventionally prepared con A to behave like intact con A toward M\(_2\). Interestingly, addition of excess Ca\(^{2+}\) and Mn\(^{2+}\) also increases the intensity of the difference spectrum generated on mixing conventionally prepared con A and M\(_1\). Similar values were observed for the change in molar absorptivity (317 nm) for the binding of both M\(_1\) (−2.9 (±0.3) × 10\(^{-4}\) cm\(^{-1}\) m\(^{-1}\)) and M\(_2\) (−3.2 (±0.2) × 10\(^{-4}\) cm\(^{-1}\) m\(^{-1}\)) to intact con A or conventionally prepared con A in the presence of ≥1 mM Ca\(^{2+}\) and Mn\(^{2+}\). The association constant for formation of a complex between conventionally prepared con A and M\(_1\) (as determined from both uv difference spectra and rate constants for association and dissociation) was observed to vary little (±15%) on changing the concentration of Ca\(^{2+}\) and Mn\(^{2+}\) from 0.1 to 5 mM. Furthermore, differences in the affinity of M\(_1\) for intact and conventionally prepared con A could not be detected at 0.1 mM Ca\(^{2+}\) and Mn\(^{2+}\).

It should be noted that although the presence of 1 mM Ca\(^{2+}\) and Mn\(^{2+}\) causes conventionally prepared con A to behave like intact con A toward M\(_2\), the metal-reconstituted proteins in conventionally prepared con A appear to be different from intact con A. For example, addition of 1 mM Ca\(^{2+}\) and Mn\(^{2+}\) to 400 μM conventionally prepared con A (pH 5, 0.5 M acetate buffer) causes this material to behave like intact con A in its interactions with M\(_2\). Ten-fold dilution of this solution to 40 μM con A, 0.1 mM Ca\(^{2+}\), and Mn\(^{2+}\) gradually causes the conventionally prepared con A to lose ability to interact with M\(_2\). After storage of this solution for 18 h at 4°C, this solution shows a spectral change, on mixing with an equal volume of 100 μM M\(_2\), almost as small as that exhibited by 40 μM conventionally prepared con A which had been dissolved directly in acetate buffer containing 0.1 mM Ca\(^{2+}\) and Mn\(^{2+}\). On the other hand, 40 μM intact con A shows no loss in its ability to interact with M\(_2\) after several days storage (at 4°C, pH 5, 0.5 M acetate buffer) in the presence of 0.1 mM Ca\(^{2+}\) and Mn\(^{2+}\). Further work is required to establish the relationship between intact con A and the metal-reconstituted, metal-deficient form of con A.

The observations that con A prepared according to the method of Agrawal and Goldstein (5) and intact con A show the same association constant 1 (±0.2) × 10\(^{-4}\) M\(^{-1}\) with M\(_1\), and that uv difference spectra of similar intensity are obtained in mixing experiments when the concentration of conventionally prepared con A and M\(_1\) are interchanged suggests, but does not prove, that M\(_1\) interacts similarly with all components in intact and conventionally prepared con A. It should be noted, however, that the low affinity of con A for M\(_1\) makes it difficult to use these spectral techniques to establish the presence of components in the solution which have different affinities toward M\(_1\), M\(_2\), which has a much greater affinity for con A than M\(_1\), and is a more sensitive probe for functional heterogeneity for con A preparations. The failure to obtain difference spectra of identical intensity in mixing experiments when the concentration of conventionally prepared con A and M\(_2\) were reversed (Fig. 1) indicates presence of components which have different affinities for M\(_2\). The lower change in absorbance under conditions of ligand excess relative to that obtained with protein excess is consistent with the presence of a component in conventionally prepared con A which binds to M\(_2\) more weakly and with a smaller change in absorbance than intact con A. This component appears to be a metal-deficient form of con A which denatures and precipitates from neutral solutions at low ionic strength. Interestingly, once the metal-deficient form of con A is reconstituted with Ca\(^{2+}\) and Mn\(^{2+}\), it exhibits the increased affinity for M\(_1\) characteristic of intact con A. At low concentrations (0.1 mM) of Ca\(^{2+}\) and Mn\(^{2+}\), however, this component is estimated to have an affinity (at pH 5.0, 1/2 0.5) for M\(_2\) (K = 2.4 × 10\(^{10}\) M\(^{-1}\)) which is similar to its estimated affinity for M\(_1\) (1 (±0.2) × 10\(^{10}\) M\(^{-1}\)).

The major difference in the interaction of M\(_1\) and M\(_2\) with intact con A is that the association constant for formation of the con A–M\(_1\) complex is 10-fold greater than that for formation of the con A monosaccharide complex. This 10-fold increase in the affinity of intact con A for M\(_1\) is difficult to explain in terms of a simple statistical effect and suggests that additional interactions with the second mannoypyranosyl residue are involved in the binding of M\(_2\), or that M\(_2\) and the monosaccharide bind to con A in different orientations. The observation that the mono- and disaccharide complexes of intact con A have identically shaped spectra (Fig. 6) and that both M\(_1\) and M\(_2\) are displaced by methyl α-D-mannopyranoside suggests that both ligands bind at the site in a similar way with their chromogenic groups in the same environment. Thus, we attribute the enhanced affinity of intact con A for M\(_2\) to simultaneous interactions between con A and groups on both mannoypyranosyl residues of M\(_2\).

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REFERENCES

15. Additional Refs. 14 to 19 are found on p. 8536
Binding of a Disaccharide to Con A

Supplement to

The effect of Con A on the binding of a disaccharide to a lectin.

Tuffy J. Williams, J. A. Staver, and T. J. Moldstein

METHODS AND MATERIALS

Con A was obtained commercially from Calbiochem. This protein was also purified using the method of Hildeman and Rabinowitz (11), and each final batch was obtained from Calbiochem. Con A was purified further from the elution fraction of a Sephadex G-100 column by aminomethylation and rechromatography on Sephadex G-100. All other reagents were Mallinckrodt, Bethesda, Mass., unless otherwise noted.

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Glycosylation constant determinations

The equilibrium constant for the association of Con A with a con A binding site (K) and the number of binding sites per protein are indicated by the equation:

\[ K = \frac{[\text{Con A}][\text{BindingSite}]}{[\text{Con A}][\text{BindingSite}]} \]

where [Con A] and [BindingSite] represent the concentrations of bound ligand, free ligand, and free protein, respectively. The number of binding sites per protein was determined by elution fraction of a Sephadex G-100 column, the binding of Con A to the original sample of Con A at equilibrium by equilibrium dialysis was determined in terms of [Con A].

The analysis of the data was performed using a computer program. The data were obtained by a computer program for comparison with the protein, which was calculated from the Sephadex G-100 column, the binding of Con A to the original sample of Con A at equilibrium by equilibrium dialysis was determined in terms of [Con A].

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Binding of a Disaccharide to Con A

Figure 1. Difference spectra obtained on mixing M and Con A obtained commercially from Calbiochem or prepared according to reference 5. λ, 40 °C, pH 7.0, and 100 cm. A1, 100 μg M, and 40 μg Con A.

Figure 2. Scattered plot for the binding of M to Con A as determined spectrophotometrically. The solid line represents a linear least squares fit of the data.

Figure 3. Normalized difference spectra for 10 μM M and Con A × (naphthol) aspiratino-phenolysis complex. The relative ammounes is defined as the ratio of the absorbance difference at a given wavelength to the absorption value of the absorbance difference at 279 nm. Spectra were obtained by mixing 20 μg of "linker" M and 50 μg ligand. A, Con M and 4, Con R-labeled N-acetylgalactosamine.

Figure 4. Scattering plot for the binding of M to Con A as determined by equilibrium dialysis. The solid line represents a linear least squares fit of the data.