The binding of p-nitrophenyl 2-O-α-D-mannopyranosyl-α-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. A second elution with 100 mM d-glucose gives concanavalin A composed entirely of intact polypeptide chains. Both fractions, which are homogeneous with respect to their interactions with M2, bind M2 with an association constant of 1.9 (±0.2) × 10^5 M^-1 at 317 nm. About 50% of the concanavalin A is contained in a third fraction which is retained on the Sephadex G-75 column. This third fraction could not be eluted from the Sephadex G-75 column by 1.0 M D-glucose, 1.0 M methyl (D-D-mannopyranoside), or 1.0 M sodium thiocyanate. The material remaining on the Sephadex G-75 column appears to be a metal-deficient form of concanavalin A. Comparison of the association constants for intact concanavalin A has a 19-fold greater affinity for M2, the metal-deficient form of concanavalin A, after being reconstituted with metal ions, appears to lose metal ions more rapidly than intact concanavalin A. Comparison of the association constants for binding of mono- and disaccharides demonstrates that intact concanavalin A has a 19-fold greater affinity for M2 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) is a carbohydrate-binding protein. The formation of a complex between con A and a carbohydrate ligand as represented by Equation 1 is a symmetrical reaction, i.e.

\[ P + 1 \overset{\text{PL}}{\rightarrow} \]

\[ \text{(1)} \]

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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 (5). 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, de-
PENDS on which component is in excess. An absorptivity
change (317 nm) of −1.6 × 10^5 cm^−1 M^−1 (based on the
concentration of con A protomer units) was determined from
the limiting change of absorbance when con A was titrated
with excess M2, whereas in absorptivity change of −3.2 × 10^5
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 absorptiv-
ities 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 attrib-
uted to: 1) impurities in the M2 preparation, 2) certain com-
ponents 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 proto-
mer-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 concen-
trations 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 inten-
sity, implying that all three preparations had the same concen-
tration 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 × 10^5 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 indi-
cate 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) × 10^5 M^−1 for K, and 0.83 (±0.2) M
binding sites per protomer unit.

Although the con A fractions which were homogeneous
with respect to binding M2 were easily displaced from the
column by D-glucose, roughly 50% of the total con A was not
eutered 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 5 M urea either in the presence or absence of 50 mM CaCl2
and MnCl2. 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) × 10^4 M^−1) of this material for M2.
The estimated amount of this material is in reasonable agree-
ment with the amount of material observed to be retained on
the Sephadex G-75 column when the commercially prepared
con A was used for the study in Fig. 2 was subjected to chroma-
tography on a Sephadex G-75 column. This result is consistent
with the hypothesis that the material remaining on the col-
umn 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. Cun-
ningham 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) simi-
lar to those obtained with conventionally prepared con A is
consistent with the view that the structural differences be-
tween the material eluted and retained on the column are
small. Our observation that solutions of conventionally pre-
pared con A, but not intact con A, develop precipitates on
standing in low ionic 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 CaCl2 and MnCl2, 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 Ca2+ and Mn2+ 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 CaCl2 and MnCl2
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 CaCl2 and MnCl2, the uv difference spectrum
is no longer altered when the concentrations of M2 and con-
ventionally prepared con A are interchanged. However, either

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When the concentration of conventionally prepared con A and Mn\textsuperscript{2+} added alone are not effective in causing conventionally prepared con A to behave like intact con A toward M\texttextsubscript{2}. Interestingly, addition of excess Ca\textsuperscript{2+} and Mn\textsuperscript{2+} also increases the intensity of the difference spectrum generated on mixing conventionally prepared con A and M\texttextsubscript{1}. Similar values were observed for the change in molar absorptivity (317 nm) for the binding of both M\textsubscript{1} (\(0.2 \times 10^4\) cm\textsuperscript{-1} m\textsuperscript{-1}) and M\textsubscript{2} (\(0.2 \times 10^4\) cm\textsuperscript{-1} m\textsuperscript{-1}) to intact con A or conventionally prepared con A in the presence of \(\geq 1\) mM Ca\textsuperscript{2+} and Mn\textsuperscript{2+}. The association constant for formation of a complex between conventionally prepared con A and M\textsubscript{1} (as determined from both uv difference spectra and rate constants for association and dissociation) was observed to vary little (\(\pm 15\%\)) on changing the concentration of Ca\textsuperscript{2+} and Mn\textsuperscript{2+} from 0.1 to 5 mM. Furthermore, differences in the affinity of M\textsubscript{1} for intact and conventionally prepared con A could not be detected at 0.1 mM Ca\textsuperscript{2+} and Mn\textsuperscript{2+}.

It should be noted that although the presence of 1 mM Ca\textsuperscript{2+} and Mn\textsuperscript{2+} causes conventionally prepared con A to behave like intact con A toward M\textsubscript{2}, the metal-reconstituted proteins in conventionally prepared con A appear to differ from intact con A. For example, addition of 1 mM Ca\textsuperscript{2+} and Mn\textsuperscript{2+} to 400 \(\mu\)M 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\textsubscript{2}. Ten-fold dilution of this solution to 40 \(\mu\)M con A, 0.1 mM Ca\textsuperscript{2+}, and Mn\textsuperscript{2+} gradually causes the conventionally prepared con A to lose ability to interact with M\textsubscript{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 \(\mu\)M M\textsubscript{2}, almost as small as that exhibited by 40 \(\mu\)M conventionally prepared con A which had been dissolved directly in acetate buffer containing 0.1 mM Ca\textsuperscript{2+} and Mn\textsuperscript{2+}. On the other hand, 40 \(\mu\)M intact con A shows no loss in its ability to interact with M\textsubscript{2} after several days storage (at 4°C, pH 5, 0.5 M acetate buffer) in the presence of 0.1 mM Ca\textsuperscript{2+} and Mn\textsuperscript{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 (\(\pm 0.2\%\)) \times 10^4 M\textsuperscript{-1} with M\textsubscript{1}, and that uv difference spectra of similar intensity are obtained in mixing experiments when the concentration of conventionally prepared con A and M\textsubscript{1} are interchanged suggests, but does not prove, that M\textsubscript{1} interacts similarly with all components in conventionally prepared con A to behave like intact con A toward M\textsubscript{2}. It should be noted, however, that the low affinity of con A for M\textsubscript{1} makes it difficult to use these spectral techniques to establish the presence of components in the solution which have different affinities toward M\textsubscript{1}, M\textsubscript{2}, which has a much greater affinity for con A than M\textsubscript{1}, 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\textsubscript{1} are reversed (Fig. 1) indicates presence of components which have different affinities for M\textsubscript{1}. 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\textsubscript{1} 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\textsuperscript{2+} and Mn\textsuperscript{2+}, it exhibits the increased affinity for M\textsubscript{2} characteristic of intact con A. At low concentrations (0.1 mM) of Ca\textsuperscript{2+} and Mn\textsuperscript{2+}, however, this component is estimated to have an affinity (at pH 5.0, 1/2 0.5) for M\textsubscript{2} (\(K = 2.4 \times 10^4\) M\textsuperscript{-1}) which is similar to its estimated affinity for M\textsubscript{1} (1 (\(\pm 0.2\%\)) \times 10^3 M\textsuperscript{-1}).

The major difference in the interaction of M\textsubscript{1} and M\textsubscript{2} with intact con A, is that the association constant for formation of the con A-M\textsubscript{2} 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\textsubscript{2} is difficult to explain in terms of a simple statistical effect and suggests that additional interactions with the second monopyranosyl residue are involved in the binding of M\textsubscript{2} or that M\textsubscript{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\textsubscript{1} and M\textsubscript{2} are displaced by methyl-\(\alpha\)-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\textsubscript{2} to simultaneous interactions between con A and groups on both monopyranosyl residues of M\textsubscript{2}.

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REFERENCES

Supplement to

**Binding of a Disaccharide to Con A**

**Taffy J. Williams, J. L. Safer,** and J. L. Holdstein

**MATERIALS AND METHODS**

Chemically purified pure Myc was available from previous studies in this laboratory. 2',5'-cyclic ADP-ribose (C-ADP-ribose) was obtained from Calbiochem. [3H]-concanavalin A was obtained from Eurogentec, Sera-Lab, UK. Other reagents were obtained from research grade suppliers.

Con A was obtained commercially from Calbiochem. This protein was also partially purified using the method of Allen and Holdstein (11). Each sample of Con A was combined and mixed in order to avoid any differences in the samples. Four hundred units of Con A were used in the assay. The solutions were heated to 55°C for 2 min and then allowed to cool to room temperature before the assay.

**RESULTS**

Sedimentation coefficients were determined for the disaccharide Con A complex. The solutions were heated to 55°C for 2 min and then allowed to cool to room temperature before the assay. The results are shown in Table 1.

**DISCUSSION**

The results show that the binding of Con A to the disaccharide is dependent on the temperature. At 55°C, the binding is strong and the complex is stable. At room temperature, the complex is more labile and the binding is weaker. The dissociation of the complex is also temperature dependent. At 55°C, the dissociation is strong and the complex is stable. At room temperature, the dissociation is weaker and the complex is more labile.

**CONCLUSIONS**

The results of this study indicate that the binding of Con A to the disaccharide is dependent on the temperature. The complex is stable at 55°C and more labile at room temperature. The dissociation of the complex is also temperature dependent. The results suggest that the binding of Con A to the disaccharide is a thermally sensitive process.
Binding of a Disaccharide to Con A

Figure 1. Difference spectra observed on binding A and A obtained commercially from Calbiochem or prepared according to references 5, 6. 40 mM HEPES, and 100 mM Con A. A, 100 mM HEPES, and 40 mM Con A.

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

Figure 4. Scanned plan for the binding of M to Con A as determined spectrophotometrically. The solid line represents a linear least squares fit of the data.
Heterogeneity of concanavalin A as detected by its binding to p-nitrophenyl 2-O-alpha-D-mannopyranosyl-alpha-D-mannopyranoside.

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