The Covalent and Three-Dimensional Structure of Concanavalin A

III. STRUCTURE OF THE MONOMER AND ITS INTERACTIONS WITH METALS AND SACCHARIDES*

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SUMMARY

The three-dimensional structure of the lectin concanavalin A (Con A) has been determined at 2.0-A resolution by x-ray diffraction analysis. The protomers are ellipsoidal domes of dimensions 42 x 40 x 39 A. Folding of the polypeptide backbone is dominated by the presence of two antiparallel pleated sheets, a twisted sheet of seven strands passing through the center of the molecule and a bowed sheet of six strands which forms the back surface of the monomer. Manganese and calcium ions bind to the protein at adjoining sites to form a binuclear complex of two octahedra sharing a common edge. The ligands for each metal ion are four groups from the NH2-terminal region of the protein and 2 water molecules. The binding site for the inhibitor p-(o-iodophenyl)-D-glucopyranoside is in a deep cavity which contains distinct hydrophobic and hydrophilic binding sub-sites. Studies of the binding of p-(o-iodophenyl)-D-glucopyranoside to Con A in the crystalline state and in solution have indicated that the binding behavior of the protein is somewhat different in the two states.

The mitogenic lectin Con A1 is a tetramer of identical protomers of molecular weight 25,500 (1, 2). Each protomer contains 237 amino acid residues (3) and has binding sites for 1 transition metal ion (nominally Mn2+), 1 Ca2+ (4, 5), and 1 saccharide (5). Co2+, Ni2+, Zn2+, and Cd2+ can replace Mn2+ at the transition metal site (6, 7). The Mn2+ ion must be bound before the Ca2+ ion, and both metal ions appear to be required for saccharide binding to occur (4, 5, 8, 9). Although transition metals can be bound in the absence of Ca2+, recent nuclear magnetic resonance measurements (10) indicate that Ca2+ has a strong influence on the rate of the Mn2+ binding process. Circular dichroism studies have indicated that upon sequential binding of metal ions and saccharide there are conformational changes affecting aromatic residues, although the gross secondary structure of the protein is apparently not affected (11, 12). Similarly, crystallographic experiments with demetalized Con A have shown that the metal-free protein is generally similar in structure to the native protein, although the geometrical relationships among the subunits are different (13).

The stereochemical requirements for the interaction between Con A and saccharides in solution have been studied extensively (4, 14-19) and n-mannose, p-glucose, and related compounds have been found to be particularly potent inhibitors of Con A. The protein apparently binds to glucosyl and mannosyl residues at the nonreducing termini of oligo- or polysaccharides (14, 19) or to certain nonterminal mannosyl residues (20). Other stereoisomers of these saccharides, such as o-galactose, apparently do not inhibit the biological activities of Con A (14).

Con A has been the subject of extensive structural investigations. Crystals of Con A have been prepared by several investigators (21-27), and details of the subunit organization have been revealed by low resolution crystallographic studies (24-27). The transition metal binding site was located through diffraction studies on crystals of Con A in which Mn2+ had been replaced by Cd2+ (7) and the binding site of the heavy atom-labeled inhibitor p-IPGlc was also located by diffraction studies (28). We have made a preliminary report of the amino acid sequence and three-dimensional structure of Con A (29). A second crystallographic study of Con A (30) was in general agreement with our results.

We report here the primary crystallographic data and a description of the structure of the Con A protomer and of its known interactions with metals and saccharides. These results are interpreted in the light of the complete chemical sequence reported in the two previous papers of this series (3, 31). In the following paper we present the atomic coordinates of Con A and an analysis of the hydrogen bonds and other noncovalent interactions involved in the stabilization of the secondary, tertiary, and quaternary structures of the protein (32).
MATERIALS AND METHODS

Concanavalin A Crystals—Con A crystallizes from 0.10 M Na2SO4, 0.01 M sodium maleate, pH 6.80, in the orthorhombic space group T222 with a = 89.9 ± 0.1 Å, b = 87.2 ± 0.1 Å, and c = 63.1 ± 0.2 Å (24, 25). The protein was isolated from defatted jack bean meal (Schwarz-Mann) by the method of Agrawal and Goldstein (8), and freed of the naturally occurring fragments (1) by NH4HCO3 precipitation of the fragments (53). Unless otherwise noted, all experiments were performed on this material. Crystals containing both intact subunits and fragments were grown from Con A that had been prepared by the method of Sunner and Howell (4). Crystals of intact subunits sometimes grew to a size of 4 × 2 × 1 mm, approximately twice the dimensions of the largest crystals grown from protein containing the naturally occurring fragments.

Isomorphous heavy atom derivatives were prepared as previously described (24, 25, 28). The conditions for the preparation of the heavy atom derivatives are shown in Table I. In three cases the conditions for preparation of derivatives have been modified since our low resolution study (24, 25): (a) Reduction of the concentration of UO2(NO3)2 from 1.0 to 0.1 mM afforded an increase in the quality of the diffraction pattern without significant reduction in the degree of heavy atom substitution, as estimated from difference electron density projections; (b) variation of the occupancy of the site designated Pt-2 with the degree of washing in platinum-free crystallization buffer permitted the preparation of two distinct K2PtCl4 derivatives; one, unwashed, with two sites of substitution; the other, extensively washed, with substitution only at the Pt-1 site; and (c) exchanging the mother liquor from difference electron density projections; (b) variation of the reduction in the degree of heavy atom substitution, as estimated from equivalent reflections on the same film and were not centered individually (34). For each reflection, the integrated peak density was measured in a rectangular area centered on the reflection. Background density was measured in four smaller rectangles arranged around the central density. The area of integration was kept constant for all reflections on a given film. Typical integration rectangles were 20 × 15 points on a 100-μm scanning raster. The illuminating aperture was 120 μm, and the receiving aperture was 100 μm. An estimated error was assigned to each intensity by

$$e(I) = E \sqrt{P^2 + (BzE)^2}$$  (1)

where E is the estimated relative error in each optical density measurement = 0.02; P, total peak density; R, total background density; R, ratio of peak integration area to background box area. Reflections within 2.4 mm of an edge of the precession pattern were omitted. A reflection was considered to be unobserved if the background-corrected intensity failed to exceed approximately two standard errors as calculated by Equation 1.

For each film pair, the film factor was estimated as ZI1/ZI1, where I1 and I2 are corresponding intensities on the front and back films. Approximately the largest 16% and smallest 1% of the observable reflections on each film were omitted from the summation. After the films of each pack were scaled together, corrections were made for Lorentz and polarization effects. No absorption corrections were applied.

**Table I**

| Derivative | Protein | Reagent | Conc (μM) | Soaking Time (days) | Overlap | Correlation R-factor | Ligand |
|------------|---------|---------|-----------|---------------------|---------|---------------------|--------|
| Pb2+       | Intact  | Pb(NO3)2 | 1         | 1                   | 11423   | .0507               | Pb1: E87, D136 (II) |
| Mersalyl   | Intact  | Sodium Mersalyl | 0.1 | 5 | 11608  | .0467 | Egl2: H127, M129 |
| Pt (unwashed) | Intact | K2PtCl4 | 1         | 7                   | 11088   | .0492               | Pt1: H127, M129 |
| Sn2+       | Intact  | Sm(NO3)3 | 10        | 1                   | 11930   | .0476               | Sm1: E87, D136 (II) |
| Pt(washed) | S-H     | K2PtCl4 | 1         | 3                   | 5110    | .0588               | Pt1: H127, M129 |
| Uranyl     | S-H     | UO2(NO3)2 | 0.1 | 5 | 4922   | .0788 | U1: DO8, NB2, DB3 |
| β-IPGlc    | S-H     | β-IPGlc | 1         | 46                  | 4719    | .0667               | U2:8 |

a "Intact" refers to protein prepared according to Cunningham et al. (33), containing purified intact subunits. "S-H" refers to protein prepared according to Sumner and Howell (4).

b Number of overlapping observations of unique reflections on different films after equivalent reflections on the same film have been averaged.

c corr = $\sum (I_{1}(h) - \bar{I}(h))/ \sum I_{1}(h)$, where I$_{1}$(h) is the observed, symmetry averaged, scaled intensity of reflection h on film 1, and $\bar{I}(h)$ is the average of the observations I$_{1}$(h).

d The Pb1 and Sm1 sites contain ligands from two different subunits. The second subunit is designated (II) as described in the following paper (32).

e Crystals used for preparation of the Pb2+ derivative were first soaked 24 hrs. in 3 M NaHCO3, 0.01 M sodium maleate, pH 6.5, to remove surface ions from the system.

f After soaking in K2PtCl4, crystals of the Pt(washed) derivative were placed in platinum-free crystallization buffer for 9 days to remove platinum from site Pt2.

g The U2 site is not associated closely with any groups of the protein, but is near H127 in the central cavity.
reactions were applied. Symmetry equivalent reflections were averaged and successively deviant observations were discarded. The average symmetry factor \( K \), defined as \( K = \frac{\sum_{\text{equiv}} I(h)}{\sum_{\text{equiv}} I(h)} \), for the native data was 0.078, where \( I(h) = \sum_{\text{equiv}} I(h) \). The average of individual intensity observations \( I(h) \) taken over all equivalent observations of reflection \( h \), and the summation is over all observations and over all reflections. Different levels were scaled together by the method of Rao (35) to form three-dimensional data sets. At this stage deviant observations were again rejected and a weight for subsequent least squares refinement was assigned to each reflection based on the larger of the observed standard error or the error assigned to the reflection by the scan-ning program. Reflections for which all observations were rejected were assigned zero weight.

Phasing and Refinement — In the calculation of MIR phases (36, 37), lack-of-closure errors for each derivative were estimated in each cycle as the average over all reflections (centrosymmetric and noncentrosymmetric) in five ranges of sin\(^2\) \( \theta \) of the errors found during the previous cycle. Probability integrals for centrosymmetric reflections are the probabilities (36) that the phase is 0 or \( \pi \), respectively. Least squares residuals were based on the squared structure factors. Centrosymmetric reflections were included from derivative, but not native, data sets when the calculated intensity was larger than the minimum observable intensity. Most probable rather than centroid phases were used in all refinements. In other respects, the phasing and refinement procedures were conventional.

The solution and refinement of the heavy atom derivatives provided the starting point for refinement at 2.8 A. Substitution sites in the K\(_2\)PtCl\(_4\) (unwashed) and K\(_2\)PtCl\(_3\) derivatives were determined by three-dimensional and projection difference Fourier techniques. The locations of heavy atom substitution were monitored by difference Fourier maps at frequent intervals during the course of refinement. Analysis of these maps indicated the presence of the atomic coordinates from the final model are described in the experimental section, knowledge of the sequence provided the assistance of protein. The sequence was particularly useful in the interpretation of the region near the natural cleavage in the Con A protein (residues 116 to 123), which was very weak in the electron density map. In the early stages of interpretation, knowledge of the sequence provided the only method of linking up segments of polypeptide chain that passed through ambiguous regions of the electron density.

The initial interpretation was recorded by placing colored adhe-sive markers at proposed atomic positions in the map. A Kendrew model was then constructed with an optical comparator (39). The solution of the model was carried out with constant refer-ence to the sequence data. The measurement and analysis of the atomic coordinates from the final model are described in the following paper (32).

Ligand Binding — The following compounds were used without further purification: \( \alpha \)-MGlc (Matheson, Coleman and Bell), \( \beta \)-PGlc and \( \beta \)-NPGal (Nutritional Biochemicals), \( \alpha \)-galactose (General Biochemicals), and \( \alpha \)-iodophenol (K and K Laboratories). \( \beta \)-IPGlc was synthesized by the acid-catalyzed condensation of \( \alpha \)-iodophenol and \( \beta \)-d-glucose pentaacetate in the Helferich reaction (41, 42), m.p. 156-158°.

\[ \text{C}_3\text{H}_6\text{O}_4 \text{I} \] (381.90)

Calculated: C 37.73, H 3.93, O 25.12, I 33.22

Found: C 37.40, H 4.12, O 25.46, I 32.98

\( \beta \)-IPGal was synthesized by reacting 2,3,4,6-tetra-O-acetyl-\( \alpha \)-L-galactopyranosyl bromide (Gallard-Schlesinger) with \( \alpha \)-iodo-

\[ \beta \text{-IPGlc was synthesized by the acid-catalyzed condensation of } \alpha \text{-iodophenol and } \beta \text{-d-glucose pentaacetate in the Helferich reaction (41, 42), m.p. 215°.} \]

\[ \text{Found: C 37.70, H 4.15, O 25.34, I 33.68} \]

The ultraviolet and infrared spectra of both \( \beta \)-IPGlc and \( \beta \)-IPGal showed characteristic features consistent with their assigned structures.

\( \alpha \)-[\( ^{13} \text{C} \)]MGlc (52.2 Ci per mol, Calitomic) was used without further purification. \( \beta \)-[\( ^{13} \text{C} \)]Glucose pentaacetate was obtained by refluxing \( \text{[\( ^{13} \text{C} \)]glucose (25 Ci per mol, Calitomic) with acetic anhydride in anhydrous sodium acetate.} \]

\( \beta \)-[\( ^{13} \text{C} \)]PIClcGlc was synthesized by performing the Helferich condensation (41, 42) between \( \alpha \)-iodophenol and \( \beta \)-[\( ^{13} \text{C} \)]Glucose pentaacetate on a microscopic scale. The final product was purified by ascending thin layer chromatography (Eastman Chromagram K301B) in butanol-acetic acid-water (4:1:5 v/v, upper phase). This compound comigrated with unlabeled \( \beta \)-IPGlc on thin layer chromatography in six different solvent systems.

The rapid "rate of dialysis" technique developed by Colowick and Womack (45) was used for the determination of the stoichiometry and association constant for the binding of various ligands to Con A. The experiments were performed in phosphate-buffered saline (1.15 g of Na\(_2\)HPO\(_4\), 0.2 g of KH\(_2\)PO\(_4\), 8.0 g of NaCl, 0.2 g of KCl per liter), pH 7.4, at room temperature using a protein concentration of about 15 mg per ml, a starting ligand concentra-tion of about \( 7 \times 10^{-4} \) M (radio-labeled) and successive additions of unlabeled ligand up to about \( 10^{-2} \) M. Values obtained for the concentrations of free [Free] and bound ligand at any given total ligand concentration are plotted according to the equation of Scatchard (46),

\[ [\text{Free}] = nK_0 - \beta K_0 \]

where \( \beta \) is the molar ratio of bound saccharide to total protein present, \( n \) is the valence of the Con A molecule, and \( K_0 \) is the association constant. The molecular weight of Con A used in these calculations was 108,000 (1, 2). Competitive binding experiments were also performed using the rapid dialysis technique by first binding ligand 1 (radio-labeled) and then displacing with ligand 2 (unlabeled). All radioactivity determinations were made by di-luting 1 ml of the sample into 15 ml of Aquosol (New England Nu-clear) before scintillation counting.

Agglutination and inhibition of agglutination assays for sheep erythrocytes (Microbiological Associates) were performed in a final volume of 250 \( \mu \)l of phosphate-buffered saline, pH 7.4, at a cell concentration of \( 1 \times 10^{9} \) cells per ml (44).
Table II
Refinement of 2.0-A data for concanavalin A

| sin² θ/λ² range | 0 to .0125 | .0125 to .025 | .025 to .0375 | .0375 to .05 | .05 to .0625 | All Data |
|-----------------|------------|--------------|--------------|-------------|-------------|----------|
| Number of reflections | 1497 | 2512 | 2574 | 2096 | 1373 | 10052 |
| Figure of merit | 0.87 | 0.77 | 0.70 | 0.64 | 0.55 | 0.71 |
| RMS Eᵃ | Pb²⁺ | Mersalyl | Pt (unwashed) | Sm³⁺ | Pt (washed) | Uranyl | β-1PGlc |
| | 66 | 66 | 70 | 54 | 84 | 93 | 74 |
| | 75 | 54 | 56 | 52 | 63 | 83 | 58 |
| | 59 | 66 | 46 | 46 | 50 | 68 | 59 |
| | 53 | 41 | 43 | 44 | 55 | 63 | 53 |
| | 50 | 44 | 43 | 44 | 55 | 63 | 50 |
| RMS fᵇ | Pb²⁺ | Mersalyl | Pt (unwashed) | Sm³⁺ | Pt (washed) | Uranyl | β-1PGlc |
| | 220 | 69 | 177 | 105 | 178 | 96 | 83 |
| | 167 | 56 | 112 | 83 | 74 | 58 | 44 |
| | 137 | 45 | 77 | 66 | 22 | 58 | 24 |
| | 116 | 37 | 57 | 56 | 17 | 46 | 13 |
| | 100 | 32 | 46 | 47 | 22 | 53 | 15 |
| | 133 | 49 | 103 | 75 | 75 | 53 | 51 |

Results and Discussion

Description of Structure—The crystallographic asymmetric unit of Con A contains one protomer of molecular weight 25,500. The protomers are compactly folded to form ellipsoidal domes of approximate height 42 Å and greatest cross section 40 × 39 Å. The domes have a somewhat smaller cross section (40 × 25 Å) at their bases. Two such domes, related by a 2-fold axis parallel to c, are joined base-to-base to form roughly ellipsoidal dimers, slightly constricted in the region of contact. The dimers are in turn paired across additional crystallographic 2-fold axes to form roughly tetrahedral tetramers of D2 symmetry. At the center of the molecule, about the 222 point, is a small pocket of solvent having some communication with the outside solution. The arrangement of the four protomers in the Con A tetramer is illustrated schematically in Fig. 1.

In our low resolution studies of Con A, we observed that the molecular surface is relatively smooth and uninterrupted, except for one large depression or cavity extending deep into each protomer. We surmised that these cavities might contain the carbohydrate binding sites of Con A and we later showed that the cavities are the site of binding of the heavy atom-labeled inhibitor β-1PGlc (28). The locations of the bound inhibitor molecules in the binding cavity of each protomer are indicated in Fig. 1, which also shows the relative locations of the essential Mn²⁺ and Ca²⁺ ions, more than 20 Å from the β-1PGlc-binding regions.

The folding of the polypeptide chain in Con A is depicted in Fig. 2, and the following discussion refers to a molecule of Con A oriented as in Fig. 2. The most striking feature of the structure is the presence of two large entirely antiparallel β structures or pleated sheets, which contain more than half the residues in the molecule (32). The large amount of β structure found is consistent with earlier circular dichroism and optical rotatory dispersion studies (11, 12, 48).

One of the pleated sheets (the "back" sheet, Fig. 3) forms almost the entire back surface of the molecule, including the rear of the β-1PGlc-binding cavity, and is associated with most of the interactions involved in dimer and tetramer formation. The plane of this sheet is bent or curled back at the top through an angle of about 30°, but it is only slightly twisted. The sheet includes a small region which turns forward from the plane of the rest of the sheet at the left in Fig. 3. The entire sheet contains about 64 residues arranged in six antiparallel chains and 9 residues in short connecting loops. The three top chains and two
Table III

Heavy metal binding of concanavalin A

| Atom | Site | Occupancy | Coordinates | Thermal parameters |
|------|------|-----------|-------------|-------------------|
| Pb1  | A    | .797      | .348 .369 .287 | 170 466 710 108 202 313 |
| Pb2  | B    | .745      | .401 .241 .177 | 522 318 6086 703 2469 5470 |
| Hg1  | C    | .302      | .553 .495 .043 | 12.1 |
| Hg2  |     | .076      | .500* .500* .030 | 30.0* |
| Pt1^e| C    | .952      | .559 .496 .058 | 1346 1231 2688 850 2386 1203 |
| Pt2^f| C    | .114      | .581 .285 .152 | 8.8 |
| Sn1  | A    | .502      | .349 .369 .288 | 30.0* |
| Sn2  |     | .363      | .707 .527 .127 | 606 903 1777 135 294 311 |
| Sm3  | B    | .128      | .401 .233 .185 | 20.0* |
| Pt1^e| C    | .988      | .559 .495 .054 | 1579 1386 2727 574 2267 1125 |
| U1   |     | .429      | .402 .259 .167 | 51.2 |
| U2   |     | .059      | .460 .500* .000* | 30.0* |
| U3   | C    | .054      | .575 .485 .057 | 30.0* |
| Il   |     | .714      | .505 .357 .197 | 47.7 |

- Sites occupied in more than one heavy-atom derivative are indicated by letters A, B, C.
- Occupancies are stated as fractions of one atom based on Thomas-Fermi-Dirac scattering factors from the International Tables for X-ray Crystallography (69) and referred to the Wilson plot scale factor of 104.4 for the native data.
- All coordinates refer to that equivalent position which is most closely associated with the protein monomer whose coordinates are given in Table I of Ref. 32. The enantiomorph has been reversed from that of Refs. 24 and 25.
- Single values are isotropic thermal parameters B (Å⁡²). Where six values are given, they are respectively the parameters B₁₁, B₂₂, B₃₃, B₁₂, B₁₃, B₂₃ in the anisotropic temperature factor expression: $[\exp(-hB₁₁ + kB₂₂ + lB₃₃ + hkB₁₂ + hkB₁₃ + kB₂₃)*10^{-6}]$.
- Unwashed platinum derivative.
- Washed platinum derivative.
- Parameters marked * were not refined.

The second pleated sheet, (the "front" sheet, see Fig. 4) extends at an angle from the upper rear of the molecule to the left front. Unlike the back pleated sheet, it is twisted in a manner...
FIG. 2. Stereo view, down the z axis, of the polypeptide backbone of a single Con A protomer, oriented with [010] horizontal and [110] approximately vertical. The Ca\(^{2+}\) and Mn\(^{2+}\) ions are indicated by CA and MN, respectively.

FIG. 3. View of the Con A protomer as in Fig. 2, but with the "back" \(\beta\) structure highlighted in black. Curling of the structure away from the vertical plane can be seen at top left. The small bent portion of the \(\beta\) structure is at far left. The loop between the two lowest chains at bottom right contains the site of the natural cleavage in Con A between residues 118 and 119.

reminiscent of the \(\beta\) structures in carboxypeptidase A (50) and carbonic anhydrase C (51). The twist amounts to about 90° in the sense of a left-handed screw along an axis perpendicular to the chain segments. The front sheet contains about 57 residues arranged in seven chains, like those of the back sheet, are antiparallel. It divides the remainder of the molecule unequally into two regions containing no regular secondary structures. The left-hand region, consisting of residues 131 to 168, is arranged in three loosely organized turns which include the front strand of the front \(\beta\) structure and part of that portion of the back \(\beta\) structure which is turned forward from the back plane. At the lower left, between this coil and the two pleated sheets, is a large internal region containing mostly hydrophobic side chains and no main chain atoms. To the right of the front pleated sheet is a region which includes the NH\(_2\) and COOH termini at the front of the molecule, the metal-binding cavity at the lower right. At the far right, just above the binding site, residues 81 to 84 comprise a single turn of approximately \(\alpha\) helical structure, the only such structure in the molecule.

Metal Binding Sites—The Mn\(^{2+}\) and Ca\(^{2+}\) ions are bound close together at the top of the molecule as shown in Fig. 2. The 2 ions are 4.6 A apart and each metal is surrounded by an approximately octahedral coordination shell containing four ligands from the protein and 2 water molecules. The metal-binding region is depicted in Figs. 5 and 6. The Mn\(^{2+}\) ion is located in the position deduced by Weinzierl and Kalb (7) from Cd\(^{2+}\) substitution experiments. The six ligands are the side chains of Glu 8, Asp 10, Asp 19, and His 24, and 2 water molecules. One of the water molecules is involved in a hydrogen-bonding network extending to the carbonyl oxygen of Val 32 and the hydroxyl oxygen of Ser 34. The other is at the inner end of a shallow depression which contains only solvent and which extends to the surface of the molecule. The observed octahedral coordination of the Mn\(^{2+}\) ion differs from the results of previous magnetic resonance (52) and diffraction (7) studies in which low Mn\(^{2+}\) coordination symmetry was suggested. However, other magnetic resonance experiments which indicate nearly cubic symmetry for the Mn\(^{2+}\) site (53), and the presence of one rapidly exchanging water ligand (54) are consistent with the observed Mn\(^{2+}\) coordination.

The coordination shell of the Ca\(^{2+}\) ion includes at least seven ligands and is less symmetrical than that of the Mn\(^{2+}\). The
FIG. 4. View of the Con A protomer as in Fig. 2 with the "front" $\beta$ structure highlighted in black. This $\beta$ structure is twisted through an angle of approximately 90\(^\circ\) from the front to the back of the molecule.

FIG. 5. Schematic illustration of the binding of metals to Con A. The Mn\(^{2+}\) and Ca\(^{2+}\) ions are both octahedrally coordinated by four protein ligands and 2 water molecules. Mn\(^{2+}\) ligands are Glu 8, Asp 10, Asp 19, and His 24; Ca\(^{2+}\) ligands are Asp 10, the backbone carbonyl of Tyr 12, Asn 14, and Asp 19. Reproduced with permission from the Ann. N. Y. Acad. Sci.

The appearance of the electron density around the Ca\(^{2+}\) suggests that the site is best described as octahedral, with one vertex occupied by both carboxylic oxygens of Asp 10. The other ligands are the side chains of Asn 14 and Asp 19, which probably contribute 1 atom each to the coordination, the carbonyl oxygen of Tyr 12, and 2 water molecules. The water molecules are in positions to be hydrogen-bonded to the carboxyl group of Asp 208 and the carbonyl oxygen atom of Arg 228 in the COOH-terminal portion of the polypeptide chain. Two of the protein ligands, Asp 10 and Asp 19, are shared by both metal ions. Thus, the entire assembly may be described as a binuclear complex composed of two polyhedra sharing a common edge (Fig. 5).

These structural features of the metal-binding region can explain some of the chemical observations concerning metal ion binding by Con A. First, most of the ligands are carboxylic acids supplied by an acidic portion of the polypeptide chain near the NH\(_2\) terminus. Protonation of these carboxylic acids would account for removal of the metals at low pH (5). Second, the site consists of the NH\(_2\)-terminal portion of the chain which is folded around both metals and includes two strands of the front $\beta$ structure. The site is completed by two portions of the chain from near the COOH terminus, one of which also belongs to the front $\beta$ structure. This arrangement, together with the requirement for sequential binding of the metal ions and the evidence

Fig. 6. Stereo drawing of the Ca\(^{2+}\)-Mn\(^{2+}\)-Con A complex. Folding of the polypeptide chain between residues 8 and 24 around the 2 metal ions is illustrated, as well as three additional pieces of chain associated with residues 32, 208, and 228, which are hydrogen-bonded to water molecules that serve as metal ligands.
that the β structure is present in the metal-free protein, suggests that in demetallized Con A, the front β structure contains a precursor transition metal binding site consisting of residues 8, 10, and 24. Binding the Mn²⁺ to this site then might induce a conformational change, bringing the connecting loop (residues 12 to 22) into a position such that residue 19 joins the Mn²⁺ coordination shell and residues 12, 14, and 19 would be in the proper orientation with respect to residues 10, 208, and 228 to create the Ca²⁺ binding site. Calcium binding would then stabilize the native conformation of the COOH-terminal portion of the chain, and possibly of the saccharide binding site. The exact details of such conformational changes must, of course, await determination of the structure of the demetallized protein.

Shoham et al. (6) have shown that the Ca²⁺ site is specific, in that it can bind Ca²⁺ and Cd²⁺, but not Ba²⁺, transition metals, or Sm³⁺. In agreement with their results, we observe that Con A crystals soaked in Sm³⁺ (Table I), Cd³⁺ (10 mM, 7 days), or Ba²⁺ (10 mM, 70 days) show no changes in electron density at the Ca²⁺ site. This specificity is in contrast to the Ca²⁺ binding sites of several other proteins. In trypsin and trypsinogen, for example, Nd³⁺ replaces Ca²⁺ (55) although Nd³⁺ does not bind to crystalline DIP-trypsin (56). Both Ba²⁺ and Nd³⁺ have been shown to replace Ca²⁺ in staphylococcal nuclease (57, 58). All 4 of the Ca²⁺ ions in thermolysin can be replaced by Ba²⁺ or Sr²⁺, and 3 of them can be replaced by lanthanides (59).

Hardman and Ainsworth (30) have described the Ca²⁺ ion in Con A as having five rather than seven or more ligands. Their description of the Ca²⁺ site differs from ours in the absence of the two water molecule ligands, in their identification of Asp 208 as a glutamic acid, and in the orientation of Asp 10. Since this site appears to be unusually special, and since penta-coordination of Ca²⁺ is extremely rare, the presence or absence of the additional ligands is of some interest. In Fig. 7 we present the electron density contours in the Ca²⁺-binding region from which we infer that both carboxylic oxygens of Asp 10 are Ca²⁺ ligands, and that a water molecule hydrogen bonded to the carboxyl oxygen of Arg 228 is also present in the coordination shell of Ca²⁺. The contours are projected approximately along a local 3-fold axis of the coordination shell. The density at the position of the additional water molecule (W5 in Fig. 7) is distinctly above the local background. The map also displays the symmetry of the Ca²⁺ site, from which we conclude that the site can best be described as an octahedron with 2 oxygen atoms at one vertex. This description is further supported by comparison of the metal-ligand bond distances and angles given in Table IV for the two metal binding sites.

In addition to the major sites of metal binding we have discussed, native Con A may bind metals at other positions. For example, we find a peak of electron density at 0.65, 0.62, 0.29, near the side chains of Glu 87, and of Asp 136 in a symmetry-related monomer belonging to the same dimer. His 180 is near enough to serve as a third ligand via a bridging water molecule. This location is the site of substitution of several metal cations used as heavy atom derivatives in the phasing calculation, including Pb²⁺ and Sm³⁺, and therefore the degree of metal occupancy in the native protein cannot be assessed reliably.

Because this peak is located on the surface of the protein and is associated with a small number of protein ligands we assume that it represents a minor metal binding site that is not necessary for the saccharide-binding activity of Con A.

Saccharides and Hydrophobic Molecules—To date, direct

![Fig. 7. Tracing of the electron density map in the vicinity of the Ca²⁺ ion with the atomic interpretation superimposed. Contours directly above the Ca²⁺ ion are omitted for clarity. Projection down the local 3-fold axis at the Ca²⁺ ion indicates the octahedral nature of the Ca²⁺ coordination shell.](https://example.com/fig7)

**Table IV**

Mn³⁺ and Ca²⁺ coordination in Con A

Mn³⁺ and Ca²⁺ positions were obtained by interpolation from the electron density; water positions by inspection of the map; and positions of protein ligands from Table I of Ref. 32. Water molecules are designated W1 to W4; protein atoms are named by combining the one-letter amino acid code, the residue number, and the conventional atom name.

| Mn³⁺-ligand distances | Ca²⁺-ligand distances |
|------------------------|------------------------|
| **Ligand** | **b(A)** | **Ligand** | **b(A)** |
| E8 OE2 | 2.9 | D10 OD1 | 2.8 |
| E8 OE2 | 2.9 | D10 OD2 | 2.9 |
| D10 OD2 | 2.7 | Y12 O | 2.5 |
| H24 NE2 | 2.4 | N14 OD | 2.1 |
| W1 | 2.5 | D19 OD1 | 3.2 |
| W2 | 2.6 | W3 | 2.4 |

**Ligand-Mn³⁺-ligand angles**

| **Ligand 1** | **Ligand 2** | **Angle** |
|--------------|--------------|-----------|
| E8 OE2 | D10 OD2 | 85.6 |
| E8 OE2 | H24 NE2 | 97.6 |
| E8 OE2 | W1 | 88.8 |
| E8 OE2 | W2 | 91.9 |
| D10 OD2 | D19 OD2 | 106.9 |
| D10 OD2 | H24 NE2 | 85.5 |
| D10 OD2 | W1 | 86.9 |
| D19 OD2 | H24 NE2 | 87.6 |
| D19 OD2 | W1 | 87.9 |
| D19 OD2 | W2 | 75.4 |
| H24 NE2 | W3 | 70.1 |
| W1 | W2 | 90.7 |
| D10 OD1 | D10 OD2 | 85.3 |
| D10 OD1 | Y12 O | 79.1 |
| D10 OD1 | D19 OD1 | 112.3 |
| D10 OD1 | W3 | 107.1 |
| D10 OD1 | W4 | 57.9 |
| D10 OD2 | Y12 O | 122.0 |
| D10 OD2 | D19 OD1 | 95.8 |
| D10 OD2 | W3 | 68.6 |
| D10 OD2 | W4 | 72.3 |
| Y12 O | N14 OD | 64.4 |
| Y12 O | D10 OD1 | 104.3 |
| Y12 O | W4 | 81.5 |
| N14 OD1 | D19 OD1 | 99.9 |
| N14 OD1 | W3 | 99.3 |
| N14 OD1 | W4 | 92.1 |
| D10 OD1 | W3 | 68.6 |
| W3 | W4 | 83.7 |
crystallographic observation of Con A-saccharide interactions has been precluded by the fact that treatment of the crystals with high concentrations of inhibitory sugars results in either dissolving of the crystals or loss of the diffraction pattern. These effects probably arise from an increase in the stability of Con A in solution (33), and a conformational change (11, 12) induced by the presence of specific saccharides. High resolution three-dimensional difference electron density maps have been made from data collected from crystals treated with the highest concentrations of several saccharides that leave measurable diffraction patterns. These maps reveal no features that can be unambiguously identified as a bound carbohydrate.

In our low resolution study we observed a large cavity in the surface of the Con A molecule (24, 25) and hypothesized that concentrations of several saccharides that leave measurable inhibition of Con A-mediated agglutination of erythrocytes and precipitation of polysaccharides (60). A 2.8-A resolution, three-dimensional difference electron density map of the $\beta$-IPGlc derivative described in Table I reveals no detectable binding of $\beta$-IPGlc at any other site in the molecule.

During the course of these studies, several lines of evidence suggested that the binding of saccharides by Con A might differ in solution and in the crystal (61–64, see below). To investigate this possibility, we carried out parallel studies of the binding behavior in solution for comparison with the crystallographic data. In agreement with the crystallographic results, these solution studies (Fig. 8) show that, within experimental error, Con A binds 4 molecules of $\beta$-IPGlc per tetramer at pH 7 and therefore that 1 $\beta$-IPGlc is bound per protomer. Similar data were obtained for the binding of $\alpha$-MGlc (Fig. 8). The association constants for the binding of $\alpha$-MGlc and $\beta$-IPGlc to Con A are $1.6 \times 10^7$ and $8.1 \times 10^5$ M$^{-1}$, respectively. These studies are in good agreement with those reported by Goldstein et al. (15, 64). Additional weak binding sites ($K_a < 10^5$) probably not be detected in our experiments.

The specificity of binding of $\alpha$-MGlc and $\beta$-IPGlc were tested by competition experiments with a variety of unlabeled compounds. Con A bound $\alpha$-[14C]MGlc can be displaced by $\beta$ IPGlc and $\beta$-PGlGlc but not by galactose, $\beta$-NPGal, or $\beta$-IPGal. A striking parallel was found between the ability of a saccharide to compete with Con A-bound $\alpha$-[14C]MGlc and the capacity to inhibit the agglutination of erythrocytes by Con A. While the glucose-containing saccharides inhibited agglutination, the galactose-containing sugars did not. The binding of $\beta$-[14C]-IPGlc can be completely competed with $\alpha$-MGlc (Fig. 9) but not with galactose, $\beta$-NPGal, or $\beta$-IPGal. A striking parallel was found between the ability of a saccharide to compete with Con A-bound $\alpha$-[14C]MGlc and the capacity to inhibit the agglutination of erythrocytes by Con A. While the glucose-containing saccharides inhibited agglutination, the galactose-containing sugars did not. The binding of $\beta$-[14C]-IPGlc can be completely competed with $\alpha$-MGlc (Fig. 9) but not with galactose, suggesting that the interaction with the protein in solution is through specific binding at the saccharide binding site. Furthermore, when solutions of Con A and $\beta$-[14C]-IPGlc are saturated with $\alpha$-MGlc, no further release of $\beta$-[14C]-IPGlc can be detected by addition of unlabeled $\beta$-IPGlc (Fig. 9) . These results indicate that in solution there is no secondary site in Con A that binds hydrophobically substituted carbohydrates but not unsubstituted monosaccharides.

In agreement with our conclusion that $\beta$-IPGlc binds to Con A with the same stoichiometry and specificity as simple glucosides and mannosides, studies of the binding of another phenyl sugar, $\alpha$-NPMan, to Con A at pH 5.55 also yielded a stoichiometry of one phenyl sugar per protomer of Con A (65). In addition, it has been shown that $\alpha$-NPMan bound to Con A can be displaced by $\alpha$-MMan (66) in a fashion similar to the displacement of $\beta$-IPGlc by $\alpha$-MGlc. Finally, recent data have shown that $\beta$-IPGlc competes on an equimolar basis with $\alpha$-NPMan (64). All of these results indicate that 1 molecule of either phenyl-substituted or unsubstituted saccharides is bound per monomer of Con A.

The region of the Con A molecule where $\beta$-IPGlc is bound in the crystal is a deep cavity which extends from the lower right edge of the molecule (Fig. 2) toward the molecular center. The back of the cavity is made up of the large $\beta$ structure which extends across the rear of the molecule (Fig. 3), while the front is delineated by the second $\beta$ structure (Fig. 4), the short helical section at residue 81, and a few sections of random coil. The cavity contains two distinct subsites with different characteristics. The first subsite is a large, predominantly hydrophobic region in which the indole atom of $\beta$-IPGlc is observed to bind (Fig. 10). This region is surrounded by the side chains of Tyr 54, Leu 81, Leu 85, Val 89, Val 91, Phe 111, Ser 113, Val 179, Ile 181, Phe 191, Phe 212, and Ile 214. The second subsite is a more constricted region between the first subsite and the molecular surface which contains predominantly hydrophilic groups.

![Equilibrium dialysis data for the binding of $\beta$-[14C]-IPGlc and $\alpha$-[14C]MGlc to Con A plotted according to the method of Scatchard (46). The experiments were performed at pH 7 and room temperature by the technique of Colowick and Womack (45).](http://www.jbc.org/)

![Competition by unlabeled $\alpha$-MGlc with $\beta$-[14C]IPGlc for binding to Con A. The experiment was performed at pH 7 and room temperature using the technique of Colowick and Womack (45). Initially, $\beta$-[14C]IPGlc ($4.1 \times 10^{-4}$ M) was added to a solution of Con A ($1.8 \times 10^{-4}$ M). After equilibration, stock solutions of $\alpha$-MGlc were added to displace Con A-bound $\beta$-[14C]IPGlc as assayed by the rising level of radioactivity in the effluent chamber. The concentration of $\alpha$-MGlc at the points indicated by the upward vertical arrows were 0.001, 0.02, 0.78, 1.8, 2.9, and 7.0 mM, respectively. Finally, unlabeled $\beta$-IPGlc was added to a final concentration of $8 \times 10^{-4}$ M to chase any residual $\beta$-[14C]IPGlc bound to the protein.](http://www.jbc.org/)
such as the side chains of Tyr 54, Ser 56, Asn 82, Ser 113, and Ser 189, as well as main chain oxygen atoms associated with Lys 114 and Ile 181 (Fig. 11). Although the glucoside ring of β-IPGlc cannot be located in the difference electron density map, it is reasonable to assume that it might be bound in this second, hydrophilic subsite.

It has been observed that a variety of nonpolar molecules such as o-iodophenol,5 o-iodobenzoic acid, and β-IPGal (62) are also bound to crystalline Con A with their respective iodine atoms in the large cavity where the iodine of β-IPGlc was located. These compounds are bound with approximately the same binding strength as β-IPGlc. These results, as well as competition experiments between β-IPGlc and α-MGlc using nuclear magnetic resonance techniques (61, 63), have led to the suggestion that the specific saccharide binding site must be outside the cavity and that β-IPGlc must be bound by two distinct sites in each protomer, one specific for the carbohydrate moiety, and one for the hydrophobic iodophenyl group (62, 63).

The binding data discussed above, however, indicate that in solution each Con A protomer binds only one saccharide, phenyl-substituted or unsubstituted. In addition, the observed orientation of the iodophenyl moiety of β-IPGal (62) indicates that the galactose ring may be bound in the inner, hydrophobic region of the cavity (to the left of the iodine in Figs. 10 and 11) and therefore cannot block the hydrophilic region (Fig. 11) near the iodine of β-IPGlc which has been proposed as the saccharide binding site. Thus, the binding of hydrophobic molecules and β-IPGal do not exclude the possibility that the saccharide binding site is within the cavity.

Preliminary data6 indicate that Con A in solution does not bind hydrophobic molecules such as o-iodophenol to any detectable extent. In addition, β-IPGal does not displace α-NPMan from Con A in solution, while α-MMMan does (64). The apparent difference in ability to bind nonpolar molecules suggests that there may be significant structural differences, at least in the hydrophobic binding region of the molecule, between Con A in solution and in the crystalline state. These differences, as well as nuclear magnetic resonance measurements of the Mn²⁺-saccharide distance to be discussed below (61, 63), raise the possibility that the interactions between Con A and β-IPGlc are significantly different in the two states.

The available data can be interpreted in terms of at least two models. One model assumes that Con A has two distinct binding sites in the two states, a saccharide-specific site unrelated to the

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\* J. L. Wang, unpublished observations.
\(\beta\)-IPGlc-binding cavity, which binds \(\beta\)-IPGlc and other inhibitory saccharides in solution but not in the crystal, and a predominantly hydrophobic site in the \(\beta\)-IPGlc-binding cavity which is not present in solution. The relative activities of the two sites are altered on crystallization either by a conformational change or by blockage by adjacent molecules in the crystal lattice. \(\beta\)-IPGlc would be capable of binding to either site, depending on the state of the protein. In the second model, the protein has a single binding region, the cavity, which contains two binding subsites, a saccharide-specific site adjacent to a hydrophobic binding site. In solution, the saccharide-specific site is most significant but the hydrophobic subsite is sufficiently strong to account for the fact that certain saccharides bearing hydrophobic aglycones are bound more strongly than the corresponding simple saccharides (60, 67). On crystallization, however, a conformational change may enhance the binding strength of the hydrophobic subsite and reduce the influence of the saccharide-specific subsite, or the saccharide-specific subsite may simply be unobservable because of the instability of the crystals in the presence of saccharides.

Attempts to locate the saccharide binding site in solution by nuclear magnetic resonance techniques have yielded conflicting results. From a study of \(^{13}\)C line broadening induced by the Mn\(^{2+}\) ion in Con A, it has been deduced that \(^{13}\)C-enriched \(\alpha\) and \(\beta\)-methyl-\(\alpha\)-glucopyranosides (61, 63) and \(\beta\)-IPGlc (63) are bound 10 to 12 A from the Mn\(^{2+}\), and therefore cannot be located in the \(\beta\)-IPGlc-binding cavity we have observed. These results have been confirmed by natural abundance \(^{13}\)C magnetic resonance spectroscopy (68). However, a more recent study,\(^7\) considering the effects on saccharide proton resonances of both the Mn\(^{2+}\) ion and of an added Gd\(^{3+}\) ion bound at Glu 87 (the Sm1 site, Table III), suggests that the saccharide binding site must be in the cavity where \(\beta\)-IPGlc is bound. Until these conflicting results can be reconciled, the magnetic resonance data must be considered inconclusive.

The available data of all kinds are insufficient to exclude either the one-site or the two-site model. Both the resonance data of Drewer et al. (61, 63) and the fact that saccharide binding is dependent on a metal-induced conformational change suggest that the saccharide binding site in solution is near the metal-binding region, favoring the two-site model. On the other hand, there are several considerations that favor the one-site model. First, the stoichiometry of \(\beta\)-IPGlc binding in both solution and crystal requires that if there are two independent sites, both must bind \(\beta\)-IPGlc strongly, but one must not exist in solution and the other must not exist in the crystal. Such a combination of changes appears unlikely. Second, careful examination of the Con A model indicates that the possible saccharide binding subsite in the cavity adjacent to the iodine of \(\beta\)-IPGlc would be capable of displaying the observed saccharide-binding specificity of Con A (Fig. 11). Several other regions on the surface of the molecule contain polar groups which might be capable of binding saccharides, but these regions are not as readily consistent with the detailed requirements of the binding specificity. Third, binding of specific saccharides inhibits the denaturation of fragmented Con A (33). So the proposed binding region in the cavity contains several residues near the point of cleavage in fragmented Con A, it is possible that a bound saccharide molecule could contribute to the stability of cleaved monomers. Finally, saccharides bearing a hydrophobic aglycone at C-1 are bound to Con A more strongly than the corresponding unsubstituted sugars (60, 67), suggesting that there is a hydrophobic subsite adjacent to the saccharide binding site in solution, consistent with the presence of the saccharide site in the cavity.

The instability of Con A crystals in the presence of saccharides and the apparent differences between the saccharide-binding activities of Con A in solution and in the crystal suggest that conclusive identification of the saccharide-specific binding region may require crystallographic studies on another crystal form of Con A or an application of other techniques such as affinity labeling. Results of such studies, in combination with the structural data presented here, should provide an explanation for the binding specificity of the protein and make possible a detailed analysis of its interactions with cell surface glycoprotein receptors.

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