Sequential Structural Changes upon Zinc and Calcium Binding to Metal-free Concanavalin A

J Julie Bouckaert, Freddy Poortmans, Lode Wynn, and Remy Lorist

From the Laboratorium voor Ultrastructuur, Vlaams Interuniversitair Instituut voor Biotechnologie, Vrije Universiteit Brussel and Vlaamse Instelling voor Technologisch Onderzoek, Boeretang 200, B-2400 Mol, Belgium

The lectin concanavalin A (ConA),¹ the lectin isolated from Canavalia ensiformis (jack bean), has for decades been known and applied as a mitogen, interacting with cell surfaces by binding specific carbohydrates (Sharon and Lis, 1989). The lectin reversibly binds saccharides only when the calcium-binding site S2 is occupied (Kalb and Levitzki, 1968). Binding of calcium itself is conditional on the binding of one of several divalent metal ions (Mn²⁺, Co²⁺, Ni²⁺, Cd²⁺, or Zn²⁺) (Shoham et al., 1973) or, at neutral pH, also Ca²⁺ (Koenig et al., 1978) at the transition metal binding site S1. The metal ions in S1 and S2 are only 4.2 Å apart and are bridged by two aspartate carboxyl groups (Asp¹⁰ and Asp²⁰⁸) (Hardman et al., 1982). Two ligand side chains of calcium are involved in monosaccharide binding, either directly (Asn⁶⁴) or via a water molecule (Asp²⁰⁸) (Derewenda et al., 1989). The binding of the metal ions causes large structural changes that pull the ligand residues of the saccharide-binding site into place and induce the carbohydrate binding capacity of the lectin. Nuclear magnetic resonance dispersion studies have allowed the successive binding to metal-free ConA of the metal ions in S1 and S2 to be followed (Brown et al., 1977). The binding of the metal ions induces a slow (from minutes to days) conformational transition in the protein, referred to as “locking” because it involves an increase in the affinity of both metals for the protein (Koenig et al., 1978). The high activation energy for this process is probably largely due to the isomerization of a non-proline peptide bond, Ala¹⁰⁷-Asp²⁰⁸, located in a β-strand neighboring the S2 site. This bond has the trans-conformation in metal-free ConA (Bouckaert et al., 1995) but adopts the unusual cis-conformation in the native protein.

A most interesting result of the NMRD studies was the demonstration that a significant portion of metal-free ConA, 12.5% at 25 °C and pH 6.4, is locked (Brown et al., 1982). Locked metal-free ConA can bind methyl-α-D-mannopyranoside with 7% of the affinity of the fully metallized locked conformation, which is at least a factor 10³ higher than its binding to the metal-free unlocked protein. Even more striking is that the unlocked/locked equilibrium can be shifted from being predominantly in the unlocked conformation to more than one-half (60%) locked, by preequilibration with excess saccharide for 5 days at 25 °C. The specificity of saccharide binding is preserved in the metal-free locked conformation (i.e. galactose does not bind). Brown and co-workers (Brown et al., 1977; Brown et al., 1982) concluded that it is predominantly the locked conformation of ConA that is responsible for the saccharide binding of the lectin. The contribution of the metal ions is to maintain the locked conformation. The binding of Mn²⁺ in S1 and Ca²⁺ in S2 shifts the unlocked/locked equilibrium completely toward the locked form.

In our work, we analyzed two structural intermediates in the pathway of the unlocked to the locked conformation, through the binding of a metal ion only in S1. A first approach was the soaking of crystals of metal-free ConA (Bouckaert et al., 1995) with zinc or cobalt (apoZn-ConA and apoCo-ConA). Zn²⁺ and Co²⁺ can bind in S1 but not in S2 (Shoham et al., 1973), in contrast to Mn²⁺, Cd²⁺, and Ca²⁺ that can occupy both sites (Shoham et al., 1973; Harrington and Wilkins, 1978; Koenig et al.

1 The abbreviations used are: ConA, concanavalin A; apoZn-ConA and apoCo-ConA, metal-free ConA crystals soaked with Zn²⁺ or Co²⁺, respectively; ConA ZnCa, co-crystals of metal-free ConA with both Zn²⁺ and Ca²⁺; EXAFS, extended x-ray absorption fine structure; Zn-ConA, co-crystals of metal-free ConA with Zn²⁺.
The binding of a metal ion in the proto-S1 site does not induce significant structural changes. Because intermolecular crystal packing interactions may hinder the sequence of changes upon metal ion binding in S1, a second approach was the co-crystallization of metal-free ConA with zinc to obtain Zn-ConA. Zn-ConA, the structure resulting from the co-crystallization of metal-free ConA with zinc to obtain Zn-ConA, has Zn$^{2+}$ bound in S1 and Ca$^{2+}$ in S2 and bears the structural features of the native, locked ConA.

### MATERIALS AND METHODS

Purification, Demetallization, Remetallization, Crystallization, and Data Collection—ConA was purified from meal of the jack bean (Canavalia ensiformis) and demetallized as described (Bouckaert et al., 1995). Throughout the crystallization experiments, systematic care was taken to prevent trace metal contamination. The solutions used for the crystallizations were batch treated with Chelex 100 beads from Bio-Rad. The pH was adjusted after equilibration with this chelator. Only metal-free pipette tips (Bio-Rad) and new plastics were used and rinsed several times with deionized water prior to use.

ApoZn-ConA and apoCo-ConA crystals originate from metal-free ConA crystals (Bouckaert et al., 1996a) soaked for 3 h in the mother liquor with 5 mM ZnCl$_2$ or CoCl$_2$. After soaking, the crystals were mounted and kept at 4°C for at least 2 weeks before data collection to allow any conformational equilibrium to be reached. The crystals remained in space group $P2_12_12_1$ of metal-free ConA and contain a dimer in the asymmetric unit (Table I).

Structural Changes in ConA upon Zn$^{2+}$ and Ca$^{2+}$ Binding

| Table I | Crystal characteristics and x-ray data |
|---------|---------------------------------------|
| ConA crystal forms | apoZn-ConA | apoCo-ConA | Zn-ConA | ConA ZnCa |
| Space group | $P2_12_12$ | $P2_12_12$ | $P2_12_12_1$ | 1222 |
| Unit cell (Å) | |
| $a$ = | 61.31 | 61.35 | 67.23 | 63.09 |
| $b$ = | 86.20 | 86.30 | 113.03 | 87.08 |
| $c$ = | 91.16 | 91.45 | 122.14 | 89.11 |
| Solvent content (%) | 45.9 | 46.1 | 43.8 | 46.9 |
| pH | 5.0 | 5.0 | 5.0 | 7.1 |
| Asymmetric unit | Dimer | Dimer | Tetramer | Monomer |
| Resolution range (Å) | 10.2-7 | 10.2-7 | 15.2-49 | 11.7-18 |
| Observations | 29303 | 31839 | 121921 | 158149 |
| Unique reflections | 12557 | 12911 | 31981 | 21738 |
| $R_{symm}$ | 8.5% | 8.0% | 11.2% | 6.2% |
| $l > 3\sigma(l)$ | 85.3 | 81.0 | 86.1 | 94.6% |
| Completeness | 91.5 | 93.8 | 97.5 | 99.4% |

---

| Table II | Refinement and geometry statistics |
|----------|-----------------------------------|
| ConA crystals | apoZn-ConA | apoCo-ConA | Zn-ConA | ConA ZnCa |
| Resolution range (Å) | 10-2.7 | 10-2.8 | 10-2.5 | 10-1.8 |
| R-factor | 20.42 | 20.70 | 19.78 | 17.61 |
| $R_{free}$-factor | 24.93 | 25.94 | 28.41 | ND* |
| No. of protein atoms | 3409 | 3409 | 7036 | 1809 |
| No. of solvent atoms | 38 | 38 | 249 | 141 |
| Deviations from ideality | |
| r.m.s.d. bond lengths (Å) | 0.019 | 0.019 | 0.029 | 0.009 |
| r.m.s.d. bond angles (°) | 2.385 | 2.338 | 2.687 | 1.669 |
| r.m.s.d. dihedrals (°) | 27.820 | 27.980 | 28.034 | 27.357 |
| r.m.s.d. improper (°) | 1.783 | 1.782 | 2.346 | 1.471 |
| Distribution of non-Gly, non-Pro ψ angles in Ramachandran plot | |
| % in most favored regions | 84.9 | 85.7 | 85.1 | 87.5 |
| % in additionally allowed regions | 13.8 | 13.6 | 14.0 | 12.5 |
| % in generously allowed regions | 1.3 | 0.8 | 0.9 | 0.0 |
| % in disallowed regions | 0.0 | 0.0 | 0.0 | 0.0 |
| Overall expected error in the coordinates (Å) | 0.25-0.35 | 0.25-0.35 | 0.25-0.35 | 0.15-0.25 |

---

* ND, not determined.  
* r.m.s.d., root mean square.  
* Degrees.
try 5CNA) (Naismith et al., 1994). A unique solution was found with a correlation coefficient of 67.6% and an R-factor of 39.2% after rigid body refinement. Individual atomic positions and temperature factors were refined using all reflections between 10 and 2.5 Å. The non-crystallographic symmetry within the dimer was strongly restrained (500 kcal/mol refined using all reflections between 10 and 2.5 Å). The non-crystallographic symmetry restraints were somewhat relaxed (300 kcal mol$^{-1}$ Å$^{-2}$) for all of the residues not involved in metal binding, saccharide binding, or packing interactions. In the last two refinement cycles, the non-crystallographic symmetry restraints were somewhat relaxed (300 kcal mol$^{-1}$ Å$^{-2}$) and applied to all of the residues, except to those that could not be included in all four monomers in the asymmetric unit (Protein Data Bank entry 1ENQ).

The space group of ConA ZnCa crystals is I222, and they are isomorphous to all ConA crystals solved up to now that contain both a transition metal ion and a calcium ion. Data were collected up to 1.8 Å. All of the data were used in the refinement. Rigid body refinement of the ConA CdCa model (Protein Data Bank entry 1CON) without the metal ions and the water molecules decreased the R-factor to 25.8% in the 10–2.46 Å resolution range. Positonal and individual temperature factor refinement of all the reflections between 10-Å and 1.80-Å resolution caused a further decrease to 24.59%. Inclusion of the Zn$^{2+}$ and Ca$^{2+}$ ions together with 133 water molecules reduced the R-factor to 21.5% before refinement and further to 19.04% after POWELL energy minimization and restrained B-value refinement. Repeated corrections and inclusion of multiple conformations for the side chains of the residues Ser21, Val72, Thr74, Ser113, and Ser134 and a final number of 142 water molecules, including one positioned on a 2-fold axis, resulted in a further decrease to 17.61% (Protein Data Bank entry 1ENQ).

RESULTS

ApoCo-ConA and apoZn-ConA—Binding of a metal ion in the S1 site only was studied both by soaking metal-free ConA crystals and by co-crystallization in the presence of transition metal ions that, by themselves, are not capable of converting the protein to its native, locked form. In all three of these crystal structures, the Ala$^{207}$Asp$^{208}$ peptide bond, which adopts the unusual cis configuration in native ConA, remains trans. Cobalt or zinc binding alone is thus not sufficient to establish the cis-conformation of the Ala$^{207}$Asp$^{208}$ peptide bond.

Upon soaking of metal-free ConA crystals with zinc or cobalt, the structural changes around the S1 site are restricted to an adjustment of the Glu$^{8}$ side chain induced by the partial binding of the metal ion. The structures of apoZn-ConA and apoCo-ConA are highly isomorphous to that of metal-free ConA. The root mean square difference for the backbone N, C, and Ca atoms with ConA ZnCa is about 0.7 Å for both structures, identical to the root mean square difference between metal-free ConA and ConA ZnCa. The metal-binding loop (Tyr$^{12}$-Tyr$^{22}$) is too flexible to display electron density. This II-loop moves around its hinges Pro$^{13}$ and Pro$^{23}$ and extends into the solvent. The resemblance to the metal-free ConA structure is apparent from the conformation of the visible edges of the loop (Pro$^{13}$, Asn$^{14}$ and Ser$^{113}$-Tyr$^{22}$) and from the clearly defined side chain conformations of the residues residing in the adjoining β-strands (Tyr$^{12}$ and His$^{100}$) (Bouckaert et al., 1995). Also, the distant saccharide-binding loop (Thr$^{97}$-Glu$^{102}$) has a conformation identical to that of metal-free ConA (Fig. 3). The peptide nitrogen atoms of Leu$^{99}$ and Tyr$^{100}$, which directly take part in monosaccharide binding, move over 4 Å in concert with the whole loop upon depletion of the metal ions. The structure of a third loop, between the residues Pro$^{203}$ and Pro$^{206}$ and preceding the Ala$^{207}$Asp$^{208}$ peptide bond that undergoes the cis-trans-isomerization upon demetallization is again similar, besides a small translation, to this of metal-free ConA in both apoZn-ConA and apoCo-ConA.

The subsequent steps in the metallization process are schematically represented in Fig. 1. Due to the moderate resolution of the monometallized structures (Table I), only a rudimentary sketch of their ligation can be made. The atoms Glu$^{8}$ OE2, Asp$^{10}$ OD2, and His$^{100}$ NE2 are always in their ligating position. The number of Co$^{2+}$ or Zn$^{2+}$ ligands varies from three to six (Table III), depending on the number of visible water ligands.

The S1 binding site conserves the positions of the ligand residues His$^{24}$, Glu$^{8}$, and Asp$^{10}$ upon demetallization (Fig. 1a) (Bouckaert et al., 1995). Asp$^{19}$, on the other hand, loses its position as a ligand residue because of its location in the metal-binding loop that detaches from the protein surface and gains high flexibility. In the holoprotein, Asp$^{19}$ anchors the metal-binding loop and helps it to fold back over the protein by its binding to both metal ions.

In apoZn-ConA and in apoCo-ConA, His$^{24}$, Glu$^{8}$, and Asp$^{10}$ are already in position and readily ligate the transition metal ion (Fig. 1b). Fig. 2 shows that the partially bound cobalt or zinc ion does not intrude as deeply into the S1 site as is the S1 site of Zn-ConA or of the holoprotein. The metal-ligand distances are unusually large (Table III), probably because of the mutual position of the disordered ion that only partially occupies the site. The Glu$^{8}$ and Asp$^{10}$ side chains retain the orientation of the metal-free form. The rest of the metal-co-ordination sphere is formed by water molecules that occupy...
equivalent positions as the water molecules in the empty site S1 of metal-free ConA. In monomer B of the dimer in the asymmetric unit, fewer water ligand molecules can be located, possibly because of the larger structural disorder in monomer B compared to in monomer A, reflected in higher temperature factors.

Zn-ConA—The co-crystals of metal-free ConA with zinc only (Zn-ConA) show the same overall structure in the metal-binding and the saccharide-binding region as the monometallized apoZn-ConA and apoCo-ConA. The root mean square deviation for the backbone N, C, and Ca atoms between the four monomers in the asymmetric unit of Zn-ConA and the monomer of apoZn-ConA varies between 0.63 and 0.73 Å. The whole metal-binding loop of Zn-ConA is visible in the electron density for three of the four monomers (except D) in the asymmetric unit. The loop is extended and has large differences in dihedral angles relative to its conformation in the holoprotein ConZnCa (Fig. 2). Zn$^{2+}$ is bound with full occupancy in the S1 site and in the same position as the zinc ion of ConZnCa, in contrast to the apoCo-ConA or apoZn-ConA structures. Its binding leads to the native-like structure of the S1 site because it orients the side chains of its ligand residues Glu$^8$, Asp$^{10}$, and His$^{24}$ similarly to ConZnCa. Despite the binding of a metal ion in S1, the S2 site is not fully formed. The ligand residues Asn$^{14}$ and Asp$^{19}$ are not in a ligating position. The side chain of Asn$^{14}$, which serves as a calcium ligand through its amide oxygen, retains too high a mobility to be visible in the electron density.

The backbones of the saccharide-binding loops of apoZn-ConA or apoCo-ConA and Zn-ConA can be perfectly superimposed (Fig. 3), except for Tyr$^{100}$. Tyr$^{100}$ of Zn-ConA is retracted into a position intermediate between those of metal-free and native ConA, 2.3 Å from its native position. Arg$^{228}$, a saccharide-binding residue, has slightly regressed (1.5 Å) from the S2 site in relation to its position in the metal-free protein. In the

**TABLE III**

| Ligand atom | apoZn-ConA | apoCo-ConA | Zn-ConA | ConA ZnCa | Native ConA |
|-------------|------------|------------|----------|------------|-------------|
|             | A          | B          | A        | B          | C           | D          |
| NE2 His$^{24}$ | 2.54 | 2.18 | 2.82 | 2.36 | 2.01 | 1.85 | 1.92 | 2.06 | 2.11 | 2.25 |
| OE2 Glu$^8$ | 2.45 | 2.66 | 3.02 | 2.89 | 2.22 | 1.99 | 2.28 | 2.20 | 2.19 | 2.23 |
| OD2 Asp$^{10}$ | 2.94 | 2.65 | 2.72 | 2.85 | 2.12 | 2.16 | 2.02 | 2.28 | 2.11 | 2.13 |
| OW D | 2.95 | 2.11 | 2.79 |  |  |  |  |  |  |  |
| OW E | 2.69 | 3.25 |  |  |  |  |  |  |  |  |
| OW F | 2.77 |  |  |  |  |  |  |  |  |  |
| OW A |  | 2.13 | 2.08 | 2.24 | 2.30 | 2.13 |  |  |  |  |
| OW B |  | 1.82 | 1.84 | 1.89 | 2.21 | 2.26 |  |  |  |  |
| OW C |  | 2.29 | 2.47 |  | 2.23 | 2.26 |  |  |  |  |
| CN$^a$ | 6 | 4 | 5 | 3 | 6 | 6 | 4 | 4 | 6 | 6 |
| Average | 2.72 | 2.40 | 2.92 | 2.70 | 2.10 | 2.06 | 2.03 | 2.19 | 2.25 | 2.21 |
| TM$^{b}$Ca |  |  |  |  |  |  |  |  |  | 4.16 | 4.16 |

$^a$ CN, coordination number.

$^b$ TM, Transition metal ion.

**Fig. 2.** Superposition of residues Glu$^8$-His$^{24}$ of the metal-binding loop and adjacent residues. The ribbon represents the backbone of apoZn-ConA (black, interrupted at Pro$^{13}$ and continued at Pro$^{23}$), Zn-ConA (dark gray), and ConA ZnCa (white). Some residues important in metal ion ligation (see also Fig. 1) and saccharide binding (see also Fig. 3) are depicted in ball-and-stick mode (MOLESCRIPT (Kraulis, 1991)). The metal ions are denoted as spheres.
Structural Changes in ConA upon Zn$^{2+}$ and Ca$^{2+}$ Binding

**Fig. 3. Superposition of the saccharide-binding loop Gly$^{95}$, Asn$^{104}$, the cis or trans peptide Ala$^{207}$, Asp$^{208}$, and Gly$^{227}$, Arg$^{228}$.**

The ribbon represents the backbone of apoZn-ConA (black), Zn-ConA (dark gray), of the ConA-methyl-$\alpha$-D-mannopyranoside complex (light gray) and ConA ZnCa (white). Residues important in saccharide binding are depicted in ball-and-stick mode. Methyl-$\alpha$-D-mannopyranoside (MeMan) in its complex with ConA (Protein Data Bank entry SCNA) and Asn$^{14}$ (only clearly defined in the ConA-MeMan complex and in ConA ZnCa) are also shown.

The number of zinc ligands varies between four and six due to the invisibility or the absence of water molecules (Table III). Besides His$^{24}$ NE2, Glu$^{10}$ OE2, and Asp$^{10}$ OD2, three water molecules are ligands to the zinc ion (Fig. 1c). Waters OW A and OW B are in the position of the water S1 metal-ligands in ConA ZnCa and native ConA. The Asp$^{19}$ carboxylate group makes a hydrogen bond to water OW C of the zinc ion. This is most clearly seen in monomer A, hinting at an important interaction that may occur to prepare monometallized ConA for the binding of the second metal ion in S2 in monomer A of ConA ZnCa. The temperature factor for this water is 31 Å$^2$; for Asp$^{19}$, it is 56 Å$^2$.

The intermolecular packing in the Zn-ConA co-crystals is distinct from any other ConA structure known. The molecules pack in a spiral staircase motif. This arrangement avoids extensive intermolecular contacts due to the crystal lattice, since there are 36 intermolecular protein-protein contacts per monomer of Zn-ConA, as compared to 93 for the monomer of ConA ZnCa. The linear ordering of tetramers gives a different amount of conformational freedom to the metal-binding and saccharide-binding regions of every of the four ConA monomers forming the tetramer in the asymmetric unit and related by non-crystallographic symmetry. In contrast to crystals of the ConA methyl-$\alpha$-D-mannopyranoside complex, not all monomers make intermolecular lattice contacts over the whole metal-binding and saccharide-binding region. The A and B monomers make contacts with residues of the metal-binding loop but not of the saccharide-binding loop. The reverse is true for the C and D monomers. In general, the loops not involved in packing interactions are more mobile and consequently not always visible in the electron density, whereas regions that take part in crystal packing interactions can be stabilized in one conformation. In monomers A and B of Zn-ConA, this leads to interpretable electron density for the metal-binding loop. There is no severe packing stress, since these “stabilized” loops still have high temperature factors.

ConA ZnCa—Co-crystallization of metal-free ConA with zinc and calcium at pH 7.1 leads to ConA ZnCa (Table I) with a structure (at 1.8-Å resolution) quasi identical to that of native ConA (Weisgerber and Helliwell, 1993) and any of the locked structures reported recently to high resolution (ConA CdCa (2.0-Å resolution) (Naismith et al., 1993), ConA CoCa (1.6-Å resolution) and ConA NiCa (2.0-Å resolution) (Emmerich et al., 1994), except for having Zn$^{2+}$ bound in S1. Also, the zinc ligation in the S1 site of ConA ZnCa (Table III) is similar to that of Mn$^{2+}$, Cd$^{2+}$, Ni$^{2+}$, or Co$^{2+}$. This is in contradiction to reported EXAFS results (Lin et al., 1990, 1991a). The zinc ion has a regular octahedral coordination sphere, and the calcium ion is hepta-coordinated (Figs. 1d and 4; Table III).

**DISCUSSION**

Conformational Changes in Metal-free ConA upon Metal Ion Binding—In all three monometalized ConA structures analyzed in this work, the crystals of metal-free ConA soaked with Co$^{2+}$ (apoCo-ConA) or with Zn$^{2+}$ (apoZn-ConA) and the co-crystals of metal-free ConA with Zn$^{2+}$ (Zn-ConA), the lectin maintains the unlocked conformation, the predominant conformation of metal-free ConA that is incapable of binding carbohydrates. The co-crystal of metal-free ConA with Zn$^{2+}$ and Ca$^{2+}$ (ConA ZnCa) contains the locked conformation of ConA. It is thus the binding of Ca$^{2+}$ in the S2 site that induces the large conformational change from unlocked to locked with the creation of the saccharide-binding site, and that is critical for the trans to cis isomerization of the Ala$^{207}$-Asp$^{208}$ peptide bond.

The Unlocked Conformation: apoCo-ConA and apoZn-ConA—The soaking of metal-free ConA crystals with Co$^{2+}$ or Zn$^{2+}$ results in partial binding of these ions in S1 without further structural change. These structures can represent the very first step in going from metal-free ConA toward the holoprotein. Metal ion binding is readily possible due to preservation of the S1 site upon demetallization, with residues Glu$^{10}$, Asp$^{10}$, and His$^{24}$ rigidly held in metal ligand positions. The absence of Asp$^{19}$, located in the extended metal-binding loop, does not disable metal ion binding in the proto-transition metal binding site S1. The S1 site residues do not adopt the native conformation (Fig. 2) but maintain the conformation of the metal-free form. Since the soaked crystals remain isomorphous to metal-free ConA crystals (Table I), the imposed crystal packing interactions may limit the accessibility of the metal-binding sites and/or hinder conformational changes upon binding of the metal ion.

The Unlocked Conformation: Zn-ConA—In the Zn-ConA co-crystal structure, Zn$^{2+}$ is bound with full occupancy. The binding of the positively charged transition metal ion in S1 requires not only the deprotonation of His$^{24}$ but also contributes significantly to the neutralization of the metal-binding region by binding two of its negatively charged residues (Glu$^{10}$ and Asp$^{10}$) and by stabilizing the third (Asp$^{19}$) by a hydrogen bond via its water ligand. Apparently, the binding of the zinc ion in S1 facilitates the subsequent binding of Ca$^{2+}$ to the S2 ligands by
Structural Changes in ConA upon Zn\(^{2+}\) and Ca\(^{2+}\) Binding

![Image](image.png)

**Fig. 4.** Omit \(F_o - F_c\) electron densities for the metal ions Zn\(^{2+}\) in the S1 site and Ca\(^{2+}\) in the S2 site and their water ligands in ConA ZnCa.

The crystallographic structure of Zn-ConA indicates that the metal-free like conformations for the S1 site residues, the carboxylate ligand residues Glu\(^8\) and Asp\(^{10}\) have native-like conformations, except for Asp\(^{19}\) (Fig. 2). The binding of a metal ion in S1 is thus a step toward the native structure. This may explain the requirement for successive binding of the metal ions first in S1 and subsequently in S2 (Brown et al., 1977) and may also explain why a larger portion of monometallized ConA (30%) is locked compared to metal-free ConA (12.5%) (the locking of ConA with only Mn\(^{2+}\) bound in S1 is represented by the equilibrium constant \(K_{LMP}\) in the metal binding scheme of Brewer et al., 1983a).

Binding of a transition metal ion in S1 does not lead to the formation of the S2 site. The S2 ligand residues are still dispersed. Therefore, the S2 site is not very successfully prepared for the binding of calcium. Only three of the five protein ligand atoms that build up the hepta-coordination sphere of calcium are in ligating positions. These are the two carboxylate atoms of Asp\(^{19}\), perfectly oriented by the zinc ion, and the peptide carbonyl of Tyr\(^{12}\), 1 Å remote from its native position. The other two protein ligand atoms, Asp\(^{19}\) OD1 and Asn\(^{14}\) OD2, are not available. Asp\(^{19}\) is too far away (Fig. 3), despite its indirect interaction with the S1 bound metal ion, and the Asn\(^{14}\) side chain is too mobile. These structural features probably account for the extremely weak binding of Ca\(^{2+}\) (C) in the S2 site to unlocked, Mn\(^{2+}\)-bound ConA (MP), with a dissociation constant of 0.3 M at pH 6.4 and 5 °C (Brown et al., 1982) (corresponding to \(K_{LMP}\) in the metal binding scheme of Brewer et al., 1983a), which is much weaker than for the binding of Mn\(^{2+}\) in the better conserved S1 site (25 μM at pH 6.4).

The Mechanism of Metal Ion-induced Conformational Locking—The metal-free and the monometallized ConA are in conformational equilibrium with the unlocked conformation favored. Metal ions not only bind, with high affinity, to the locked form of metal-free ConA, but moreover also bind to the unlocked conformation and induce locking. The binding of Mn\(^{2+}\) and Ca\(^{2+}\) decreases the intrinsic locking time constant (at 5 °C) to 0.27 h (Brewer et al., 1983b) and shifts the unlocked/locked equilibrium completely toward the locked form.

The crystallographic structure of Zn-ConA indicates that the calcium ion can bind only weakly in the disrupted metal-binding site S2 of unlocked ConA after the binding of a metal ion in S1. The large flexibility of Ca\(^{2+}\) in accepting more variable and irregular coordination geometries than similar ions (McPhalen et al., 1991) and cooperativity among the metal-binding ligands may help the Ca\(^{2+}\) binding in S2 and lead to the formation of a fully metal-bound, but still unlocked, ConA species. To stabilize its coordination in S2, the calcium ion must induce locking and must overcome the energy barrier separating the trans and the cis isomers of the Ala\(^{207}\)-Asp\(^{208}\) peptide bond. The isomerization of this bond, ideally situated in the β-strand facing the saccharide-binding loop (Thr\(^{97}\)-Glu\(^{102}\)) on the one side and the S2 site on the other side, could be the key needed for the locking. On the one side, the isomerization leads to the active conformation of the saccharide-binding loop, thereby requiring the disruption of two strong hydrogen bonds between the side chains of Asp\(^{208}\) and Asn\(^{104}\) (Bouckaert et al., 1995; see also the orientation of these side chains on Fig. 3). On the other side, the calcium ion stabilizes the cis-conformer, and one of its water ligands makes hydrogen bonds to the carbonyl and a carboxylate oxygen of Asp\(^{208}\).

The existence of an initially unlocked state of the fully metal-bound ConA was demonstrated by Brown et al. (1977), who showed that EDTA readily removes the metals from unlocked, Mn\(^{2+}\), and Ca\(^{2+}\)-bound ConA (CMP). Moreover, this form binds methyl-α-D-mannopyranoside only 1 order of magnitude better than the metal-free unlocked form (Koenig et al., 1978). The lack of saccharide binding capacity and high dissociation rates of the metal ions, particularly in the S2 site, indicate that this fully metal-bound ConA structure is not yet locked. However, it is likely that the S1 and S2 sites are already well structured by the metal ion binding in the unlocked, fully metal-bound ConA state, because the relaxivity dispersion of the water protons by the Mn ion in the unlocked and the locked species of Mn\(^{2+}\)- and Ca\(^{2+}\)-bound ConA have almost the same value (CMP and CMPL, respectively, in Brown et al., 1977).

The mechanism of saccharide-induced locking is different from that of metal ion-induced locking. Methyl-α-D-mannopyranoside can bind only to the locked form of metal-free ConA and is not known to influence the intrinsic locking time of the transconformation event (Grimaldi and Sykes, 1975; Brewer et al., 1983b). Stabilization of the locked form by binding of the monosaccharide shifts the unlocked/locked equilibrium toward the locked form. It is not known whether the cis-conformer is established in locked, metal-free ConA. We presume that the locked form of metal-free ConA must have a well formed S2 site, because of its high affinity for both the S1 and S2 metal ions. Co-crystallizations of metal-free ConA with saccharides have been set up, thus far without results.
In conclusion, a mechanism is proposed for the conformational changes upon metal ion binding to metal-free ConA that explains the results from this crystallographic study and that is in agreement with results from nuclear magnetic resonance dispersion (Brewer et al., 1983b), fluorescence (Harrington and Wilkins, 1978), and stopped-flow nuclear magnetic resonance kinetic studies (Grimaldi and Sykes, 1975). The sequence of ConA structures indicates the requirement for the sequential binding of the metal ions firstly in S1 and then in S2, followed by the initially weak binding of Ca$^{2+}$ in the disrupted S2 site that on its turn orders S2 and the Ca$^{2+}$-induced locking that comprises the trans to cis isomerization of the Ala$^{207}$-Asp$^{208}$ peptide bond with the ensuing formation of the saccharide-binding site and the stabilization of the whole metal-binding and saccharide-binding region.

Metal Ligation in ConA ZnCa—In the structures of the holoprotein analyzed up to now, including this ConA ZnCa derivative, the nature of the S1 metal ion (Mn$^{2+}$, Cd$^{2+}$, Co$^{2+}$, or Ni$^{2+}$) has not been found to influence its overall coordination geometry (Hardman et al., 1982) or the saccharide binding. The only difference is the average transition metal-ligand distance, increasing from 2.18 Å for Co$^{2+}$, 2.20 Å for Ni$^{2+}$ (Emmerich et al., 1994), 2.21 Å for Cd$^{2+}$ bound in S1 (Weigiser and Helliwell, 1993), over 2.25 Å for Zn$^{2+}$ (this work), to 2.31 Å for Cd$^{2+}$ (Naimism et al., 1993).

The coordination number of the zinc ion in ConA ZnCa calculated from the EXAFS measurements is 6 for the solution sample but only 4 in the crystals (Lin et al., 1991a). This difference was ascribed to intermolecular crystal packing forces imposed on the residues involved in lattice contacts, transmitted to and absorbed by the zinc ion (Lin et al., 1990). The zinc ion would displace the crystal packing stress by shortening its metal-ligand distances and the release of two of its ligands. The discrepancies that exist in coordination number of the S1 bound metal ion, not only between this crystallographic study and the EXAFS study, but also between the first interpretations of EXAFS (Kalb (Gilboa) et al., 1979; Lin et al., 1990), and its later revisions (Lin et al., 1991a; Lin et al., 1991b), can probably largely be ascribed to unobserved water ligand molecules. Zinc-coordinated water molecules are kinetically labile and typically exchange rapidly (Bertini and Luchinat, 1994).

Zinc and Cobalt Binding to ConA, Their Specificity for S1, and Their Inability to Bind in S2—Why are Co$^{2+}$ and Zn$^{2+}$ unable to bind in the S2 site of ConA? The S2 site preferentially binds calcium with a low dissociation constant (about 10$^{-8}$ M in Koenig et al., 1978). Ca$^{2+}$ (ionic radius, 0.99 Å) fits S2 in hepta-coordination ion. Co$^{2+}$ and Zn$^{2+}$ have a relatively small ionic radii (0.70 and 0.71 Å, respectively, for a coordination number of 6) and average coordination numbers smaller than 6 (5.7 and 5.0, respectively) (Glusker, 1991). Cobalt and zinc may be too small and make too few contacts in the large, irregular Ca$^{2+}$-coordination sphere. The second zinc ion per ConA monomer found by Palmer et al. (1980) to displace Cd$^{2+}$ is more likely bound in the third metal-binding site distinct from the S2 site. This site is capable of binding cadmium, present as the second cadmium ion bound per ConA monomer in the crystal structure of ConA CdCa besides the S1-bound cadmium. The third metal-binding site also binds heavy metal ions like Pb$^{2+}$ and Sm$^{2+}$ (Palmer et al., 1980; Becker et al., 1975), Gd$^{3+}$, Tb$^{3+}$, and Eu$^{3+}$ (Barber et al., 1975; Sherry and Cottam, 1973).

The Zn-ConA co-crystal at pH 5 illustrates how little the binding of the S1 ion prepares the S2 site for calcium ion binding. The possibility exists that Mn$^{2+}$ or Cd$^{2+}$ bound in S1, in the absence of a metal ion in S2, leads to preferential binding of Ca$^{2+}$ rather than Zn$^{2+}$, because they are larger (ionic radius; 0.8 and 0.91 Å, respectively, in hexa-coordination) and tend to have a higher coordination number (6.0 and 6.1, respectively) (Glusker, 1991). They might interact directly with Asp$^{19}$ instead of via a water molecule. Monometalized manganese or cadmium ConA, however, are difficult to isolate, because these ions can bind in both S1 and S2 (Bouckaert et al., 1996).

Acknowledgments—Thomas Hamelryck and Jürgen Pletinckx are gratefully thanked for carefully reading the manuscript. We thank Maria Vanderveken for excellent technical assistance. We are indebted to Raymond Kemps (VITO, Mol) for metal microanalysis of the crystals. We are grateful to R. A. Palmer and J. Cooper from Birkbeck College for the data collection on ConA ZnCa.

REFERENCES

Barber, B. H., Fuhr, B., and Carver, J. P. (1975) Biochemistry 14, 4075–4082.

Bouckaert, J., Loris, R., and Wyns, L. (1995) Biochemistry 34, 9461–9472.

Brown, R. D., III, Brewer, C. F., and Koenig, S. H. (1982) Biochemistry 21, 465–469.

Bruenger, A. T. (1992) Acta Crystallogr. Sect. D 48, 886–892.

Brewer, C. F., Brown, R. D., III, and Koenig, S. H. (1983) Biochemistry 22, 3691–3702.

Brewer, C. F., Brown, R. D., III, and Koenig, S. H. (1983) Biochemistry 22, 3683–3696.

Brown, R. D., III, Brewer, C. F., and Koenig, S. H. (1982) Biochemistry 21, 465–469.

Glusker, J. P. (1991) Adv. Prot. Chem. 42, 3–76.

Hardman, K. D., Agarwal, R. C., and Freiser, M. J. (1982) Biochemistry 21, 961–976.

Hardman, K. D., Agarwal, R. C., and Freiser, M. J. (1982) J. Chem. Soc. Perkin Trans. 2, 157–163.

Kraulis, P. J. (1991) Bioinformatics 7, 405–416.

Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950.

Luzatti, P. V. (1952) J. Chem. Soc. 1513–1524.

McPhalen, C. A., Strynadka, N. C. J., and James, M. N. G. (1991) Adv. Prot. Chem. 42, 77–144.

Pflugrath, J. W., and Messerschmidt, A. (1989) Biochemistry 28, 12435–12441.

Pflugrath, J. W., and Messerschmidt, A. (1989) Biochemistry 28, 12435–12441.

Pflugrath, J. W., and Messerschmidt, A. (1989) Biochemistry 28, 12435–12441.

Raftery, J., Kalb (Gilboa), A. J., Yariv, J., Dauter, Z., and Wilson, K. S. (1994) J. Biol. Chem. 269, 2189–2193.

Sharon, N., and Lis, H. (1989) Biochemistry 28, 12435–12441.

Sherry, A. D., and Cottam, G. L. (1973) Arch. Biochem. Biophys. 156, 665–672.

Shoham, M., Kalb, A. J., and Pecht, I. (1973) Biochemistry 12, 1914–1917.

Weigiser, S., and Helliwell, J. R. (1993) J. Chem. Soc. Faraday Trans. 89, 2667–2675.