The reversible binding of manganese and calcium to concanavalin A determines the carbohydrate binding of the lectin by inducing large conformational changes. These changes are governed by the isomerization of a non-proline peptide bond, Ala-207–Asp-208, positioned in a β-strand in between the calcium binding site S2 and the carbohydrate specificity-determining loop. The replacement of calcium by manganese allowed us to investigate the structures of the carbohydrate binding, locked state and the inactive, unlocked state of concanavalin A, both with and without metal ions bound. Crystals of unlocked metal-free concanavalin A convert to the locked form with the binding of two Mn2+ ions. Removal of these ions from the crystals traps metal-free concanavalin A in its locked state, a minority species in solution. The ligation of a metal ion in S2 to unlocked concanavalin A causes bending of the β-strand foregathering the S2 ligand residues Asp-10 and Tyr-12. This bending disrupts conventional β-sheet hydrogen bonding and forces the Thr-11 side chain against the Ala-207–Asp-208 peptide bond. The steric strain exerted by Thr-11 is presumed to drive the trans-to-cis isomerization. Upon isomerization, Asp-208 flips into its carbohydrate binding position, and the conformation of the carbohydrate specificity determining loop changes dramatically.

Lectins are a structurally very diverse class of proteins that bind carbohydrates with considerable specificity but moderate affinities (1). Their ability to bind carbohydrates often depends on the binding of metal ions that mediate with the carbohydrate directly (the Ca2+-dependent animal lectins (2)) or indirectly (lectins from the Leguminosae family). Leguminosae lectins form a large family of plant lectins that succeed in covering a broad range of fine specificity toward oligosaccharides through subtle variations in length and sequence of five different loops A–E (3–5). All Leguminosae lectins share the need for transition metal ion and calcium binding to stabilize the active conformation of these loops. The five amino acids involved in metal binding are fully conserved. These are a histidine (His-24 in ConA) and a glutamate (Glu-8 in ConA) for the transition metal ion binding site S1, an asparagine for the calcium binding site S2 (Asn-14 in ConA), and two aspartate residues (Asp-10 and Asp-19 in ConA) bridging S1 and S2 by their carbohydrate groups. All these five amino acids originate from two β-strands and their connecting metal binding loop (see Fig. 2).

Carbohydrate binding by Leguminosae lectins also involves a number of conserved residues. The monosaccharide binding site contains an Asn, Asp, Gly/Arg triad, originating from three loops: the metal binding loop C, loop A that follows the conserved cis peptide, and loop B, respectively. The conserved asparagine (Asn-14 in ConA) of the triad binds carbohydrate via its amide nitrogen but also ligates calcium through its amide oxygen. The aspartate (Asp-208 in ConA) is involved in a conserved non-proline cis-peptide bond that is in turn stabilized by calcium binding in S2. The third member of the conserved triad (Arg-228 in ConA) interacts both with the carbohydrate and via a water molecule with the metal ion in S2. Furthermore, an aromatic residue in the metal binding loop is essential for carbohydrate binding. On the other hand, the carbohydrate binding residues in the carbohydrate specificity-determining loop, or monosaccharide specificity-determining loop D, and loop E (3–5) are not conserved. Neither are they involved in metal binding. All Leguminosae lectins are, thus, in the same way dependent on metal ion binding. Therefore the mechanism by which metal ion binding establishes the minimal structural requirements for carbohydrate binding merits a deeper understanding.

Most studies have focused on the metal binding properties of Con A, because demetallization of ConA is reversible, in contrast to most other Leguminosae lectins. Demetallization of other closely related leguminous lectins such as lentil and pea lectin is often irreversible and leads to precipitation of these proteins (6). As a consequence, ConA is the only family member for which crystal structures of its metal-free and partially metallized forms have been analyzed (7, 8).

The binding of metal ions to ConA occurs at two adjacent sites located at the top of the dome-shaped molecule (Fig. 1). In its native state, ConA binds a transition metal ion in site S1 and a calcium ion in site S2. The transition metal binding site (S1) of ConA preferentially binds Mn2+, but can also accommodate Ni2+, Co2+, Zn2+, Cd2+, Cu2+ (10), or even Ca2+ (11, 12). The S2 site is at 4.16 Å from S1 and binds Ca2+ but can also accommodate Cd2+ or Mn2+ (13).

Metal binding by ConA and the properties of the corresponding molecular resonances in solution have been studied in detail by Brewer et al. (for a review, see Ref. 14). Early nuclear magnetic resonance dispersion experiments indicated a high energy barrier (about 22 kcal mol−1) separating the two conformers of ConA (15). This high energy barrier and the consequently long equilibration times for the conformational change were attributed to the isomerization of the peptide bond between Ala-207 and...
Asp-208. Brewer et al. (14) conclude that ConA occurs in essentially two conformational states, which they called the locked, containing the cis peptide, and the unlocked state, containing a usual trans peptide. Both states are in equilibrium with each other, but the equilibrium constant depends on the presence or absence of metal ions. In the absence of any metal ions, the dominant species is the unlocked state. In the presence of specific metal ions, the conformational equilibrium shifts completely toward the locked state. The locked, metal-bound form of ConA corresponds to the native Mn\(^{2+}\)- and Ca\(^{2+}\)-bound ConA as it is observed in its carbohydrate-free form as well as in a number of carbohydrate complexes.

We set out to characterize by x-ray crystallography the different species on the metal binding pathways that have been observed in solution (Scheme 1, according to Brewer et al. (14)) and to follow metal ion binding and conformational conversions in the crystalline state (Scheme 2). To do so, we made use of the observation that Mn\(^{2+}\) can functionally replace Ca\(^{2+}\) (14, 15). In the absence of Ca\(^{2+}\), Mn\(^{2+}\) binds to S2. Mn\(^{2+}\) in the S2 site has a much larger dissociation constant (\(K_d = 25 \mu M\) at pH 6.4) than Ca\(^{2+}\) (\(K_d = 0.3 \mu M\) at pH 6.0) and is in rapid equilibrium with the solvent (11). As such, it can be much more easily extracted from the protein. Equally important for our work was the observation that when Mn\(^{2+}\) is used as a replacement for Ca\(^{2+}\), the conversion from the unlocked to the locked form is slowed down significantly, occurring on a time scale of hours or even days (at 5 °C) instead of minutes (14). This allowed us to collect x-ray data on intermediates that occur only transiently or as minority species in solution.

### EXPERIMENTAL PROCEDURES

Production of Crystals of Unlocked Metal-free ConA (U ConA). Unlocked Mn\(^{2+}\)-bound ConA (UMn ConA), and Locked Double Mn-bound ConA (LMnMn ConA)—ConA was purified, demetallized, and crystallized as described previously (7). One crystal of the resulting metal-free ConA in the unlocked form (U ConA) was used to collect a 2.05-Å resolution data set on beam line BW7B of the DESY synchrotron, Hamburg, Germany. These data were merged with low resolution data previously collected on our home source (7). UMn ConA was produced by soaking a single crystal of U ConA for 1 h in the mother liquor to which 7 mM MnCl\(_2\) had been added. The crystal was subsequently mounted, and data were collected immediately after, using a rotating anode generator. To prepare LMnMn ConA, crystals of metal-free ConA were soaked for 3 h in 60 \(\mu l\) of the mother liquor at pH 5 with the addition of 5 mM of Mn\(^{2+}\), resulting in LMnMn\(^{2+}\) ConA. The crystals were then mounted in borosilicate capillaries and stored at 4 °C for at least 2 weeks. Crystals of LMnMn ConA were also produced by co-crystallization of metal-free ConA and Mn\(^{2+}\), resulting in LMnMn\(^{2+}\) ConA. These crystals were grown in sitting drops of 75 \(\mu l\) composed of 20 \(\mu l\) of 17.3 mg/ml metal-free ConA, 50 \(\mu l\) of reservoir solution (2 mM Na acetate, 0.1 M NaCl, 0.1 M Na phosphate, and pH 6.0) at 20 °C. In the absence of any metal ions, the equilibrium constant depends on the presence or absence of metal ions. The horizontal equilibria are values for the dissociation constants of Mn\(^{2+}\) from the Mn\(^{2+}\) complexes (at 25 °C for the binding of Mn\(^{2+}\) in S1, at 5 °C for the binding of Mn\(^{2+}\) in S2). The upper and lower vertical equilibria are dissociation constants for methyl-\(\alpha\)-d-mannose (S) binding, and the middle vertical equilibria are defined as the ratios of the respective equilibria of unlocked to locked species. The values are measured or deduced.
ions and to ensure that the sample was not contaminated by trace manganese for calcium, since no distinction can be made between Ca$^{2+}$ and Mn$^{2+}$. For this reason, the sample was subjected to soaking experiments using Chelex-100 beads before use to prevent any contamination of the protein with trace metals. After data collection, the metal content of every crystal was checked on a scanning electron microscope with an energy-dispersive x-ray analyzer to ensure the presence of the required metal ions and the absence of undesired ones. An anomalous data analysis has been performed on L MnMn ConA crystals. In the case of U ConA, synchrotron radiation was used (beam line BW7B of the DESY synchrotron), whereas all other data were collected using a Rigaku RU200 rotating anode CuK$_\alpha$ radiation. All regular data were collected using a MAR image plate detector data, integrated using DENZO, and scaled using SCALEPACK (16), with the exception of the data of LMnMn ConA collected on a FAST detector and processed using MADNESS. The anomalous data of L MnMn ConA were scaled using SCALA (17) and subsequently scaled and merged using ROTAVATA and AGROVATA (18). The statistics of the data collections are shown in Table I.

**Structure Determination**—The structures were solved by molecular replacement when necessary. Rigid body refinement and subsequent positional and individual B-factor refinements were performed without cut-off on the data using X-plor (19), except for U ConA, which was refined with the aid of CNS (20). Starting coordinates for refinement were the 2.5-Å structure of metal-free ConA (Protein Data Bank code 1apn (7)) for the unlocked structures and the 1.85-Å structure of LznCa ConA at pH 7.1 (Protein Data Bank code 1enr (8)) for the locked structures. Except for L MnMn ConA, which was refined earlier, the refinement was started with a high temperature (3000 K) slow-cool stage to remove model bias and to uncouple the crystallographic R-factor and the R-free factor. The refinement was evaluated by cross-validation using R-free (21), and a bulk solvent correction was applied and updated throughout the refinement. For the addition of the metal ions, no metal-ligand bond distance restraints were employed, and the metal ions were given no charge. Water molecules were added only if 1) the water had at least one hydrogen bonding partner, 2) the positive difference density level was higher than 3 $\sigma$, and 3) the electron density of the water reappeared after refinement in the 2 F$_o$ – F$_c$ map at a level of at least 1 $\sigma$. The final refinement statistics are shown in Table I.

**Metal Ion Identification**—To confirm the nature of the bound metal ions and to ensure that the sample was not contaminated by trace amounts of, mainly, calcium during crystallization or crystal handling, the identity of the metal ions in the co-crystals of L MnMn ConA was evaluated by means of anomalous dispersion of the Mn$^{2+}$ ions. Identification of the metal ions was a prerequisite to prove the substitution of manganese for calcium, since no distinction can be made between Ca$^{2+}$ (18 electrons) and Mn$^{2+}$ (23 electrons) on the basis of the electron density. Manganese has its K-absorption edge at the x-ray wavelength of 1.896 Å. The anomalous dispersion experiment was performed, however, at the wavelength of 1.5418 Å of Cu K$_\alpha$ radiation, widely used in laboratory x-ray sources, in combination with a MAR area detector. Data of L MnMn ConA were collected using the inverse beam strategy, and Bijvoet mates were treated separately to calculate anomalous difference Patterson and Fourier maps. An anomalous-difference Patterson map has been calculated with the subroutine HASSP of the program HEAVY (22) by means of single atom, two atoms, and cross-peak searches.

**RESULTS**

**Composing a Structural Repertoire via the Reversible Binding of Non-native Metal Ions**—Starting from crystals of metal-free or U ConA, we attempted to produce and characterize by x-ray crystallography the different metal-bound and metal-free species observed by Brewer et al. (14) in solution (Scheme 1). The ConA species that could be produced by a successive number of experiments involving the addition or removal of metal ions from crystals of ConA are shown in Scheme 2. The statistics for the data collection and refinement of each of these crystals are summarized in Table I. In the case of the U Mn ConA, data to only 2.9 Å could be collected because of the transient nature of this species, which spontaneously converts to L MnMn ConA. Therefore, short data collection times were necessary, although U MnMn ConA crystals diffract only weakly. It was not possible to flash-freeze the U MnMn ConA crystals, as earlier freezing experiments had already indicated that this inevitably leads to contamination with Ca$^{2+}$. Consequently all crystals were exposed to x-rays at room temperature.

Crucial to the isolation of the transient intermediate U Mn ConA was the use of Mn$^{2+}$ as a substitute for Ca$^{2+}$ in S2; first, because the addition of both Mn$^{2+}$ and Ca$^{2+}$ to U ConA crystals results in cracking of the crystals, and second, because the binding of Mn$^{2+}$ instead of Ca$^{2+}$ in S2 decreases the time constant of the locking process from about 1 min to 0.5 h in solution at 25 °C (14). The U Mn ConA species ultimately converts to L MnMn ConA. A third advantage of using only Mn$^{2+}$ is that Mn$^{2+}$ can be extracted from the L MnMn ConA crystals due to the weak binding of Mn$^{2+}$ to S2 (Scheme 1). This allowed the production of crystals of L ConA, which occurs only as a minor species in solution (14) (Scheme 2).

**U ConA and UMn ConA**—The crystal structure of metal-free ConA (also called apo-ConA or demetalized ConA) has recently been described by us at a resolution of 2.5 Å (7). To obtain a more detailed picture of this conformational state of ConA, we used synchrotron radiation to collect data to 2.05 Å, the maximum resolution observed on beam line BW7B of the DESY synchrotron. The structure corresponds to the predominant unlocked species or U ConA found in solution and confirms the results of our previous studies on metal-free ConA. As in the earlier 2.5-Å resolution structure, the disordered metal binding loop Pro-13-Tyr-22 is not visible in the electron density map.

Soaking of crystals of U ConA with MnCl$_2$ initially leads to the binding of a single Mn$^{2+}$ ion in the S1 site if the soaking time is sufficiently short and data are collected immediately afterward. The structure of the U Mn ConA complex is essentially identical to those in Uzn ConA and UCo ConA that were obtained in the same way and that have been described in detail before (8). Changes compared with U ConA are minor and restricted to the metal binding sites. In contrast to Uzn and UCo ConA, UMn ConA is not a stable entity. In the time course of 2 weeks at 4 °C, it spontaneously converts to L MnMn ConA. UMn ConA also contains an extra Mn$^{2+}$ ion bound to Asp-80 and Asp-82 at a crystal lattice contact. This binding site has not been observed in any ConA structure before, but is not considered to be of functional importance.

**Locked Double Mn$^{2+}$-bound ConA (LMnMn)**—When crystals of U ConA (space group P2$_1$2$_1$2; $a \sim 60$ Å, $b \sim 85$ Å, $c \sim 91$ Å) are soaked with MnCl$_2$ for a longer period of time, they go through a disordered phase showing blurred reflections. After 2 weeks, the diffraction quality of the crystals has improved dramatically, and their space group and unit cell have changed to those of the native crystal form of ConA (I222; $a \sim 63$ Å, $b \sim 87$ Å, $c \sim 89$ Å). During this transition, the ConA tetramers in the unit cell have rotated 14° relative to each other, and ConA has transformed from the unlocked to the locked form, with...
Mn$^{2+}$ ions bound in both sites S1 and S2. The structural changes corresponding to locking are complex and involve the trans-to-cis isomerization of the crucial Ala-207–Asp-208 peptide bond and the structural reorganization of a large portion of the protein (7). The resulting structure, LMnMn\textsuperscript{PH5.0} ConA, is virtually identical to that of LMnCa ConA. The acidity of the crystallization solution did not prevent locking and allowed the conversion to space group I222. The conformation of the metal binding loop Pro-13–Tyr-22 and of the monosaccharide specificity site Thr-97–Glul-102 loop are thus largely pH-independent in the presence of an excess of specific metal ions.

LMnMn ConA that was prepared in solution from U ConA could also be crystallized at pH 7. LMnMn\textsuperscript{PH7.0} ConA is iso morphous with LMnMn\textsuperscript{PH5.0} ConA obtained by soaking of U ConA crystals. Therefore we can conclude that the transformation from unlocked to locked in the crystal is identical to that in solution. When we compare LMnMn ConA with LMnCa ConA, only minor distortions were found in the direct neighborhood of the metal ions (Table II). Equally at pH 5 or pH 7, we found slightly larger metal-ligand distances and a significantly larger intermetal distance. These are in agreement with a poorer stabilization of the metal binding sites and the larger dissociation constant of Mn$^{2+}$ from the S2 site (Scheme 1 (11)). In the structure at pH 5, the temperature factor of Mn$^{2+}$ at S2 is slightly higher than at S1, whereas in all other fully metal-bound ConA structures close to pH 7, the opposite is true (Table II). This is probably due to the lower binding constant of Mn$^{2+}$ in S2 at pH 5 compared with at pH 7.

\textbf{Mn$^{2+}$ Identification by Anomalous Diffraction—}\textit{The Mn$^{2+}$ ions of LMnMn\textsuperscript{PH7.0} ConA show up unambiguously as the two highest peaks in the anomalous Patterson map, and their positions coincide exactly with the metal ion positions in the crystal structure of ConA. This implies that identification of manganese using CuK\textalpha radiation was possible on the short wavelength side, even far away from the absorption edge of the anomalous scatterer, although a difference in intensity of the reflections of only 3.83 electrons (23) is calculated for the single Mn$^{2+}$ ion in LMnMn ConA. Data collected in an identical manner for our LMnCa ConA crystals, with a theoretical intensity difference of 2.11 electrons due to the single Mn$^{2+}$ ion, did not reveal the position of the Mn$^{2+}$ ion in the Patterson map. Kalb \textit{et al.} (24) recently succeeded in localizing the peaks for this single Mn$^{2+}$ ion of LMnCa ConA in the anomalous Patterson maps by data collection at three different wavelengths (24); at the inflection point of the absorption edge, at the peak absorption point, and at a remote wavelength. Anomalous difference Fourier maps of the LMnMn and LMnCa ConA co-crystal structures display the Mn$^{2+}$ ions as the highest peaks (Fig. 2). In LMnMn ConA, the peak height ratio of the Mn$^{2+}$ ion to S1 is compared with Mn$^{2+}$ bound to S2 is 1.44. In the anomalous-difference Fourier maps of LMnCa ConA, Ca$^{2+}$ in S2 appears as the second highest peak with a ratio of Mn$^{2+}$ over Ca$^{2+}$ of 2.23 (theoretically 2.18 at 1.5418 Å (23)). This further confirms the presence of Mn$^{2+}$ in the S2 site of LMnMn\textsuperscript{PH5.0} ConA, in agreement with nuclear magnetic resonance dispersion experiments (14) and electron paramagnetic resonance and magnetic susceptibility studies (25).

\textit{L ConA—}Crystals of LMnMn\textsuperscript{PH7.0} ConA have been soaked in their crystallization liquor that was saturated with EDTA. Over a time course of 2 weeks, this resulted in the slow extraction of the Mn$^{2+}$ ions from both S1 and S2. The Mn$^{2+}$ ion in S2 is extracted faster than that in S1. Refinement against the data collected at the different time points shows intermediate crystal structures that contain a mixture of LMnMn, LMn, and L ConA (data not shown). After 3 days of soaking, the data suggest a L Mn ConA structure that is virtually identical to the LMnMn

| TABLE I X-ray data collection and refinement statistics of the six ConA structures |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Protein Data Bank code | U pH 5.0 | LMn pH 5.0 | LMnMn pH 5.0 | LMnMn pH 7.0 | L pH 7.0 | LCaCa pH 7.0 |
| Species | U pH 5.0 | LMn pH 5.0 | LMnMn pH 5.0 | LMnMn pH 7.0 | L pH 7.0 | LCaCa pH 7.0 |
| Detector | MAR IP | MAR IP | MAR IP | MAR IP | MAR IP | MAR IP |
| Data analysis software | DENZO | DENZO | MADNESS | DENZO | DENZO | DENZO |
| Unit cell | a (Å) | 60.50 | 60.89 | 63.23 | 63.20 | 63.01 | 62.96 |
| | b (Å) | 84.23 | 85.01 | 87.44 | 87.41 | 87.29 | 87.31 |
| | c (Å) | 91.02 | 91.62 | 89.27 | 89.30 | 88.81 | 89.94 |
| Space group | P2\textsubscript{1}2\textsubscript{1}2 | P2\textsubscript{1}2\textsubscript{1}2 | P2\textsubscript{1}2\textsubscript{1}2 | P2\textsubscript{1}2\textsubscript{1}2 | P2\textsubscript{1}2\textsubscript{1}2 | P2\textsubscript{1}2\textsubscript{1}2 |
| Resolution | 10.0–2.05 | 8.0–2.9 | 8.0–2.9 | 8.0–2.9 | 8.0–2.9 | 8.0–2.9 |
| Number of observed reflections | 68,956 | 45,995 | 48,708 | 90,119 | 311,946 | 262,158 |
| Number of unique reflections | 26,108 | 11,615 | 19,435 | 19,671 | 311,946 | 262,158 |
| Highest resolution shell 2.10–2.05 | 76.3 (54.8) | 76.3 (54.8) | 76.3 (54.8) | 76.3 (54.8) | 76.3 (54.8) | 76.3 (54.8) |
| Reflections with I > 3\sigma(I) (%) | 74.9 (48.7) | 74.9 (48.7) | 74.9 (48.7) | 74.9 (48.7) | 74.9 (48.7) | 74.9 (48.7) |
| R-value | 0.092 | 0.092 | 0.092 | 0.092 | 0.092 | 0.092 |
| R-free value | 0.279 | 0.279 | 0.279 | 0.279 | 0.279 | 0.279 |
| Bulk solvent correction | Yes | Yes | No | No | Yes | Yes |
| r.m.s. bond lengths (Å) | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 |
| r.m.s. bond angles (degree) | 1.5 | 1.6 | 3.6 | 3.6 | 3.6 | 3.6 |
| r.m.s. dihedral angles (degree) | 25.9 | 27.1 | 28.3 | 28.3 | 28.3 | 28.3 |
| r.m.s. improper (degree) | 0.8 | 1.5 | 2.0 | 1.7 | 1.5 | 1.5 |
| Ramachandran plot (non-Gly, non-Pro) | Most favorable | 82.9 | 80.6 | 88.0 | 88.5 | 88.9 | 86.5 |
| | Additionally allowed | 16.1 | 19.2 | 12.0 | 11.5 | 11.1 | 13.5 |
| | Generously allowed | 0.8 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 |
| | Disallowed | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Number of water molecules | 144 | 35 | 106 | 125 | 184 | 86 |
structure, except for the Mn\(^{2+}\) ion in S2, which may be substituted by a water molecule as in L ConA (see below).

After soaking with EDTA for 2 weeks, a locked structure is obtained that essentially lacks any metal ions in S1 and S2 L ConA. The crystals of the metal-free L ConA did not convert to the space group of metal-free U ConA. This situation did not change after prolonged soakings in EDTA over a period of more than 6 months with regular refreshment of the soaking solution. Also, a gradual decrease in pH of the soaking solution did not induce such a change. Clearly the I222 space group provides the more stable crystal packing, as observed in the crystal structures, and prevents L ConA from unlocking. In this respect, crystal packing may functionally mimic carbohydrate binding, which also shifts the conformational equilibrium toward the locked form (14). Indeed, the crystal lattice interactions stabilizing the locked state involve the carbohydrate binding site in its functional conformation. The L ConA structure is very similar to LMnCa ConA or LMnMn ConA. Differences between L ConA and LMnMn ConA are restricted to the metal binding sites. Water residues 303 and 311 are bound in S1 and S2, respectively (Figs. 3 and 4). They are displaced 0.5 Å and 0.4 Å, respectively, from the metal ion positions in S1 and S2 in LMnCa ConA. This contrasts with the situation in U ConA, where no water is found on the metal ion position in the partially formed S1 site. The small difference in placement of the water molecules compared with the metal ion allows them to form normal-length hydrogen bonds (2.5 Å or longer) with the water molecules that use to be first shell metal ligands. The side chain of Asp-19, one of the S1,S2 bridging carboxylate groups, shifts by more than 1 Å. This narrows the S1 site, thus expelling one of the two water molecules that normally ligates the metal ion in S1 and makes a hydrogen bond with the second shell metal ligand, the Ser-34 hydroxyl group (Fig. 3).

Calcium Binding to L ConA—

In the locked conformation, both the S1 and S2 metal binding sites of metal-free ConA are preformed and consequently can rapidly take up metal ions. This enabled us to investigate the nature of a locked species that contains only calcium and no transition metal ion (Scheme Table II)

| S1       | U Mn1 pH 5.0 | U Mn2 pH 5.0 | LMnMn pH 5.0 | LMnMn pH 7.0 | LMnCa pH 6.5 | CaCa pH 7.0 |
|----------|--------------|--------------|--------------|--------------|--------------|-------------|
| Metal ion| Mn\(^{2+}\) (44) | Mn\(^{2+}\) (45) | Mn\(^{2+}\) (15) | Mn\(^{2+}\) (15) | Ca\(^{2+}\) (33) |
| Glu-8 OE2| 2.74 (53)    | 2.95 (52)    | 2.21 (8)     | 2.23 (14)    | 2.15 (27)    |
| Asp-19 OD1| 2.83 (40)    | 2.90 (56)    | 2.22 (7)     | 2.19 (15)    | 2.50 (28)    |
| His-24 NE2| 2.47 (31)    | 2.07 (15)    | 2.26 (13)    | 2.46 (41)    |              |
| Water O  | 2.31 (49)    | 2.17 (46)    | 2.24 (6)     | 2.28 (14)    | 2.40 (34)    |
| Water O  | 2.30 (26)    | 2.40 (18)    | 2.13 (9)     | 2.48 (57)    |              |
| Average  | 2.63 (47)    | 2.67 (51)    | 2.27 (12)    | 2.21 (12)    | 2.37 (35)    |
| S2       |              |              |              |              |              |
| Metal ion| Mn\(^{2+}\) (30) | Mn\(^{2+}\) (13) | Ca\(^{2+}\) (10) | Ca\(^{2+}\) (38) |
| Asp-19 OD2| 2.24 (31)    | 2.56 (14)    | 2.30 (11)    | 2.56 (36)    |
| Asp-10 OD2| 2.54 (33)    | 2.55 (7)     | 2.45 (15)    | 2.26 (28)    |
| Asn-14 OD1| 2.26 (31)    | 2.22 (8)     | 2.28 (10)    | 2.36 (33)    |
| Tyr-12 O | 2.64 (28)    | 2.48 (15)    | 2.55 (15)    | 2.89 (37)    |
| Water O  | 2.52 (35)    | 2.11 (15)    | 2.37 (10)    | 2.53 (39)    |
| Water O  | 2.52 (35)    | 2.45 (15)    | 2.32 (12)    | 2.64 (26)    |
| Average  | 2.41 (39)    | 2.39 (12)    | 2.37 (12)    | 2.50 (33)    |
| S1–S2 distance | 4.34 | 4.32 | 4.16 | 4.25 |

Fig. 2. Anomalous-difference Fourier electron density in the S1 and S2 sites of LMnMn ConA (a) compared with LMnCa ConA (b). Carbon atoms are in black, nitrogen atoms are in blue, oxygen atoms are in red. The metal binding loop is shown, together with the conserved metal ligand protein residues (Glu-8, Asp-10, Asn-14, Asp-19, and His-24), metal ligand water molecules (purple), the Ala-207–Asp-208 dipeptide in ball-and-stick model, and second shell metal ligand residue Ser-34. The displayed electron density is brought to a same level in the S1 site that contains manganese in both structures to enable comparison of the electron density in the S2 sites (levels of electron density are shown in green, light blue, dark blue, and red).
2). Such a species is known to exist from solution studies (11), but it remained unclear whether calcium-only occupies S2 or both sites. However, the soaking of L ConA crystals in a solution containing calcium leads to the instant filling of both metal binding sites. Compared with L ConA, the side chain of Asp-19 reorients to coordinate the calcium ions correctly and, thus, allows the second ligand water molecule to enter the S1 site. No further conformational changes are involved, as the protein was already locked. The coordination geometry of the calcium ions in S1 and S2 is as usual, except for slightly enlarged metal-ligand distances (Table II).

Comparison of the Locked and Unlocked State, Independent of Metal Ion Binding—The repertoire of structures studied enables us to distinguish the large differences between the unlocked and locked conformational states from the local metal ion binding-related differences. The structural features characterizing the locked and unlocked states count for all locked versus unlocked structures and are independent from the metal-bound state of the protein. All residues affected by locking and unlocking belong to just four β-strands (Fig. 1) and their connecting loops. These β-strands are Ile-4–Asp-10, preceding the metal-binding loop, Pro-206–Asn-216, including the famous Ala-207–Asp-208 peptide, and Trp-88–Thr-97 and Thr-103–Ser-117, preceding and following the monosaccharide specificity loop D. The Ile-4–Asp-10 strand, with Thr-11 at its edge, and the adjacent strand between 206 and 210, move about 1 Å between the locked and unlocked conformation of ConA (Fig. 5c). Binding in S2 changes the conformation of the Asp-10 side chain and of the metal binding loop with the S2 ligands Tyr-12 and Asp-19 (Fig. 5) and initiates the locking process. The bending of the β-strand Ile-4–Asp-10 over the S2 site upon binding of a metal ion in S2 by the Asp-10 and Tyr-12 ligands (Table II) leads to the loss of two conventional main chain–main chain β-sheet hydrogen bond interactions between Gly-209 and Ser-96. In unlocked ConA, the carboxylate group of Asp-208 fits in between the β-strands Pro-206–Asn-216 and Pro-209–Ser-210.
Thr-103–Ser-117. On the side of the monosaccharide specificity loop, Asp-208 makes two hydrogen bonds with the amide side chain group of Asn-104. Toward S2, the main chain of Asp-208 makes a hydrogen bond with the Thr-11 hydroxyl group. These interactions in unlocked ConA are replaced in locked ConA by interactions via side chains only, of Asp-208 with Thr-11, Glu-102, and Asn-104, located two β-strands apart (Fig. 1). The Glu-102 carboxylate group takes in the place of the Asp-208 carboxylate group (Fig. 5c). Similar to Asp-208 in unlocked ConA, Glu-102 makes hydrogen bonds in two directions. Toward the S2 site, it interacts with the Thr-11 hydroxyl and toward the Thr-103–Ser-117 strand adjacent to the monosaccharide specificity loop, and it interacts with the amide group of Asn-104.

**DISCUSSION**

**Impact of the Metal Ion Substitutions on the ConA Structure**—We confirmed, primarily by anomalous dispersion of the Mn$^{2+}$ ion, the earlier observations of Brown et al. (15) that Mn$^{2+}$ or Ca$^{2+}$ alone can form a stable complex with ConA by the occupation of both the S1 and S2 sites. Binding of the natural S2 ion, calcium, in S1 is allowed with conservation of the usual coordination geometry. Binding of the natural S1 ion, Mn$^{2+}$, in S2 is weak (Scheme 1) and leaves a partial disorder in the binuclear metal ion binding site. This is visible in the LMnMn ConA structures, both at pH 5 and pH 7, and confirmed by the enlarged intermolecular S1-S2 distances. The most plausible explanation for this inefficiency is that the ionic radius of Mn$^{2+}$ (ionic radius is 0.8 Å in hexa-coordination) is rather small compared with Ca$^{2+}$ (0.99 Å) or Cd$^{2+}$ (0.91 Å) (10) and close to the limit for being accepted as a substrate in the S2 site. Still smaller ions like Zn$^{2+}$ and Co$^{2+}$ (0.71 Å and 0.70 Å, respectively) cannot bind in S2 but only in S1 (8). These reasons allow the removal of Mn$^{2+}$ from LMnMn ConA crystals by dialysis.

**The Trapping of an Unstable Species, L ConA, in the Crystal**—Our present crystallographic studies together with our previous work (7) confirm several major aspects of Scheme 1, such as the switch between two major conformational species involving a cis-trans isomerization of the Ala-207–Asp-208 peptide bond and the existence of a locked ConA species in the absence of metal ions. In solution, only about 13% of metal-free ConA is locked under the given conditions. It is thus unlikely to spontaneously grow L ConA crystals. However, L ConA is transiently produced when Mn$^{2+}$ ions are extracted from LMnMn ConA in solution by EDTA (14), making use of the weaker binding of Mn$^{2+}$ to S2 compared with Ca$^{2+}$. When this method was applied to LMnMn crystals, L ConA crystals are produced that are stable. The opposite case, the conversion from UMn to LMnMn, is slowed down but not prevented by the crystal lattice, congruent with the space group conversion from P2$_1$2$_1$2$_1$ to I222. L ConA is prevented from unlocking due to the tight crystal packing interactions in the metal ion and carbohydrate binding region in the I222 space group and the highly cooperative nature of the crystal lattice. If these crystal packing forces would be undone as for the protein in solution, the region in L ConA may have a somewhat different structure that, however, must still be functional, according to Scheme 1.

**Connecting Structure to Biochemistry**—The existence of L ConA allows us to study the two major conformational states, locked and unlocked, independently from the direct metal ion binding differences. Our crystallographic data show that all the locked structures, whether metal-bound or not, turn out to be very similar in structure. The same is true for the series of ConA structures in the unlocked state. The changes within these two groups of structures are restricted to small differences in the metal binding region. In contrast, the unlocked and locked states differ over a much more extended region of the protein (Fig. 1).

Production of L ConA crystals also allowed us to recognize another unstable species predicted from solution studies, namely LMn ConA (Scheme 1). This species originates from the slow and incomplete extraction of Mn$^{2+}$ from LMnMn ConA.
crystals by soaking in EDTA. No removal of Mn$^{2+}$ was observed after 1 day, and only partial removal was observed after 3 days. Data collected after 3 days revealed that the removal of Mn$^{2+}$ from S2 is faster than removal of Mn$^{2+}$ from S1, congruent with the dissociation constants displayed in Scheme 1 and leading to a mixture of L ConA and LMn ConA. These data do not show a significant movement of the Asp-19 side chain, in contrast to the L ConA data collected after complete removal of metal ions. Taking all our crystal structures together, the existence of all but one of the saccharide-free unlocked and locked forms in Scheme 1 is confirmed. However, the existence of the missing transient UMnMn ConA species is required to reconcile structure and biochemistry.

**The UMnMn ConA Species**—Of all the different species identified by Brewer et al. (14) (Scheme 1), only one is still of unknown structure. This is the unlocked form containing metal ions bound in both S1 and S2. In the UMn ConA structure, the manganese in S1 is not bound at full occupancy, as was also observed for zinc and cobalt in the stable UZn and UCo complexes (8)). Given that Mn$^{2+}$ binding in S2 of the unlocked form is still an order of magnitude weaker than in S1, congruent with the dissociation constants displayed in Scheme 1 and leading to a mixture of L ConA and LMn ConA. These data do not show a significant movement of the Asp-19 side chain, in contrast to the L ConA data collected after complete removal of metal ions. Taking all our crystal structures together, the existence of all but one of the saccharide-free unlocked and locked forms in Scheme 1 is confirmed. However, the existence of the missing transient UMnMn ConA species is required to reconcile structure and biochemistry.

**Proposed Mechanism of Non-proline cis-peptide Isomerization**—Binding of a metal ion in S2 is the driving force to initiate the locking process. In the UMn ConA structure, Mn$^{2+}$ binding in the S2 site may then transiently generate UMnMn ConA, after which the equilibrium shifts toward the locked form. **Locking and Unlocking Are Conformational Changes in a Concerted Fashion**—It was not possible to follow the locking process directly in a time-resolved study because of the inherent disorder of the diffraction patterns and the change of space group during the transition. The question thus remains whether the pathway involves a single co-operative step or involves further intermediates. We therefore constructed mosaic models for the unlocked-to-locked transition and vice versa by the combination of structural elements of the unlocked and locked states (Fig. 6). None of these models are physically plausible structures due to steric clashes. This is suggestive of a concerted mechanism in which the conformational changes occur almost simultaneously with the isomerization of the Ala-207–Asp-208 peptide bond. The model explaining the unlocked-to-locked transition contains structured S1 and S2 sites in combination with the trans Ala-207–Asp-208 peptide and the monosaccharide specificity loop of the unlocked state (Fig. 6a).

**ConA Metal Ion Binding and Peptide Bond Isomerization**

![Fig. 6. Mosaic models combining the conformational features of the locked and the unlocked state of ConA.](http://www.jbc.org/)

The backbone is colored black for the unlocked state and white for the locked state. a, with structured metal binding sites as in metal-bound ConA and a trans Ala-207–Asp-208 peptide bond, a clash is generated between the side chain of Thr-11 and the backbone of the Ala-207–Asp-208 peptide. b, the combination of the metal binding sites of unlocked ConA with a cis Ala-207–Asp-208 peptide lacks the interactions that induce and maintain the cis peptide.
looking, by pulling Asp-10, Tyr-12, and Asp-19 together and produces the structured binuclear metal binding site of the locked form (Fig. 6a). The β-strand Ile-4–Asp-10 bends over S2 to ligate the metal ion, thereby distorting the conventional main chain to main chain β-sheet hydrogen bonding between this strand and the adjacent strand Pro-206–Asn-216 (Figs. 5, a and b) and pushing the side chain of Thr-11 against the Ala-207–Asp-208 peptide bond (Fig. 6c). Thr-11 makes a steric clash with Asp-208 (2.02 Å between Thr-11 OG1 and Asp-208 O, 2.30 Å between Thr-11 OG1 and Asp-208 Cβ). A hydrogen bond may, however, form between Thr-11 N and Asp-208 O (distance 2.6 Å). We suggest that the release of steric stress between Thr-11 and the Ala-207–Asp-208 peptide backbone drives the trans-to-cis isomerization. Due to the isomerization, the Asp-208 side chain turns around the backbone and toward the monosaccharide binding site, whereupon the side chain of Glu-102 comes in to substitute for those interactions of the Asp-208 carbohydrate group lost upon isomerization (Fig. 5c). The interactions of the β-strand Trp-88–Thr-97 and Thr-103–Ser-117 on the side of the cis peptide away from the S2 site, and the conformation of the monosaccharide specificity loop Thr-97–Glu-102, change dramatically by the isomerization event.

Going from the locked to the unlocked state, metal ions are first released to produce the L ConA structure. Consequently the β-strand Ile-4–Asp-10 detaches from the S2 site and straightens up (Fig. 6b). This leads to the loss of the hydrogen bond interaction between the Thr-11 hydroxyl and the Glu-102 carboxylate (distance is 4.4 Å). In our model, the side chain of Thr-11 sterically hinders the edge of the β-strand at Pro-206. The carbonyl group of Asp-208 comes into close contact with Ca and Cα of Asp-10. Most importantly, the space between residues Asp-10–Tyr-12 and Pro-206–Asp-208 is opened up by the straightening of the β-strand Ile-4–Asp-10. The energetically demanding cis peptide is thus destabilized, and the structure tends to collapse and refold with a usual trans Ala-207–Asp-208 peptide.

Finding Specific Reasons for Carbohydrate Binding by Unlocked ConA—Interestingly, the peptide bond isomerization or locking of ConA can also be triggered by carbohydrate binding instead of by calcium binding. The addition of methyl-α-D-mannose to metal-free ConA in solution, containing about 13% L ConA under the given conditions, increases the amount of L ConA to 60% (14). This could be due to the preferential binding of saccharide to locked ConA, because carbohydrate binding to unlocked ConA could only be attributed a very low affinity (Scheme 1). The key polar interactions of the carbohydrate side chain of Asp-208 with the carbohydrate O4 and O6 hydroxyl groups are impossible in U ConA. Binding of carbohydrate to fully metal-bound but unlocked ConA is, however, not possible, in agreement with the observation of a weak complex between mannose and UMnMn ConA (Scheme 1). We suggest that the reason for this weak binding is the dictation of many direct monosaccharide-protein interactions by the structure of the binuclear metal binding site. When a metal ion is bound in S2, the carbohydrate ligand atoms of Asn-14 and Arg-228 are oriented correctly to interact with the hydroxyl groups O4 and O3, respectively, of the monosaccharide. On the other hand, the monosaccharide specificity loop of UMnMn ConA does not have the right conformation to sterically and specifically restrict the binding. Carbohydrate binding to UMnMn ConA is, thus, largely limited by the lack of the specific interactions with the residues of the monosaccharide specificity loop as well as by the lack of coordinated hydrogen bonds by Asp-208.

Has the Isomerization of the Peptide Bond a Physiological Meaning?—Many of non-proline cis-peptide-containing proteins are carbohydrate binding or carbohydrate-processing (27). Non-proline cis-peptide bonds are considered functionally important (27, 28), as has been very recently demonstrated for two scorpion toxins (29). Conformational changes with loss of sugar binding activity during endosomal transit of the mannose and asialoglycoprotein receptors has been suggested to depend on a pH-regulated loss of calcium (30). These changes include a prolyl peptide isomerization as a possible mechanism to limit the rate of gain and loss of ligand binding activity during processes such as receptor-mediated endocytosis and, thus, to facilitate sorting of carbohydrate-bearing ligands from the receptor in the endosome (31).

Leguminosae lectins could be endowed with a similar mechanism of pH-dependent release of the calcium ion. The seed lectins may function as a packaging aid for storage proteins and glycosidases into developing protein bodies (32). They bind other seed proteins mostly by ionic interactions that are optimal between pH 5 and 6, close to the conditions in imbibed seeds and developing protein bodies (33). A small portion of storage proteins and the protein body membrane specifically binds to the seed lectins via its carbohydrate binding site (34, 35). Toward the acidic side, the carbohydrate-mediated interaction falls off steeply. We propose that this may have to do with the loss of the metal ions. The weakening of interaction could allow the lectins to set free the storage glycoproteins during imbibition of the seeds. The slow kinetics of the non-proline cis-trans isomerization could withhold the lectins from rebinding the storage glycoproteins before diffusing out of the seedlings, in which vicinity they can contribute to the protection against bacterial, fungal, and viral pathogens (36) after regaining activity in the less acidic and calcium-rich soil.

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