Changes in the three-dimensional structure of concanavalin A upon demetallization

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Contributed by Gerald M. Edelman, March 10, 1978

ABSTRACT  When the Mn2+ and Ca2+ ions normally present in concanavalin A are removed, the protein becomes incapable of binding saccharides. To explore the structural differences between the native and demetallized forms, and their effects on the saccharide-binding properties of the protein, we have refined and compared the crystal structures of both forms. Refinement, carried out by automated difference Fourier methods, has revealed a number of differences between the two structures as well as minor differences between the two crystallographically independent monomers in the demetallized structure. Significant differences between the holo- and apoproteins are confined to the region where the metals are bound. These differences include a reorganization and disordering of the loop, consisting of residues 7-25, that contains all of the direct metal ligands of the protein. In some molecules, the side chain of arginine-523 appears to move into the metal-binding region, possibly compensating in part for the absence of the metals' positive charge. The Cis peptide observed in the native protein at alanine-207 is apparently not present in the demetallized protein. The conformational differences affect many of the residues currently thought to be involved in the specific binding of saccharides.

The lectin concanavalin A (Con A) contains two metal ions (1, 2) that are required for the specific saccharide-binding and biological activities of the protein (3, 4). The metal ions are bound to the protein 4.5 Å apart in two octahedral sites, each of which comprises four protein ligands and two water molecules (5). Two of the protein ligands, Asp10 and Asp19, are shared by the two sites. The first site, designated S1, binds a transition metal ion, usually Mn2+. S1 must be occupied before the second site, S2, can bind its metal ion, usually Ca2+ (3). The two ions stabilize the formation of a specific saccharide-binding site approximately 10-13 Å from the metals (6-8). An unusual cis peptide linkage has been identified in the region of the metal- and saccharide-binding sites (9, 10).

Spectroscopic data indicate that conformational changes accompany the binding of both metals (11, 12) and saccharides (12, 13), and the demetallized (apo-) protein, native (holo-) protein, and protein–saccharide complexes all crystallize in different space groups. We are carrying out high-resolution crystallography studies on each of these structural variants of Con A. The three-dimensional structure of the native form was the first to be determined (5, 14), and it has now been refined (unpublished results). The location of the saccharide-binding site was established in a 3.5-Å-resolution study of a crosslinked protein–saccharide complex (7) and in a 6-Å-resolution study of a different complex (8), but the detailed mode of saccharide binding is not yet known. We have previously reported the determination of the structure of demetallized Con A at 2.8 Å (7), and another study of this structure has been carried out independently (cited in ref. 15 as unpublished work). We here present further details of the structure solution, a preliminary account of the refinement of the demetallized structure, and a comparison of the demetallized and native structures with emphasis on the conformational changes that accompany the addition and removal of the metals.

MATERIALS AND METHODS

Data Collection. Con A was prepared (4, 16) and demetallized (17), and the demetallized protein was crystallized (18), by standard procedures. The crystals are in the orthorhombic space group P212121, a = 92.00, b = 85.91, c = 61.52 Å, with two Con A protomers in the asymmetric unit. Diffraction data were recorded to approximately 2.8-Å resolution on screened precession photographs and the films were processed as described (19). The average symmetry R-factor based on intensities for the 50 films in the data set was 0.0987, and the overall R-factor for film-to-film scaling was 0.0849. No absorption correction was applied.

Solution of the Structure. The structure of demetallized Con A was solved by molecular replacement methods (20). Rotation functions were calculated by the fast Fourier transform method (21), typically with data between 6- and 20-Å resolution and a radius of integration in Patterson space of 35 Å. “T1” translational functions (22) using data to 4-Å resolution were used to determine the origins of the Con A subunits. Difference electron density maps were calculated with coefficients \( r[F_o] - \sum_s F_e \exp(i\alpha_s) \) and \( 2k [F_o] - |F_o| \exp(i\alpha_o)/|F_o| = \) observed structure factor magnitude, \( |F_o| = \) calculated structure factor magnitude, \( \alpha_o = \) calculated phase. Initially, scale factors, \( k \), were calculated in 10 equal ranges of \( |F_o| \) to minimize \( \sum |F_o| - |F_o| \) by \( \sum \) and each coefficient was weighted by \( \exp(-k |F_o|^2 - F_o^2)/2E^2) \) to reduce the contribution of poorly estimated structure factors. After refinement, a single overall scale factor was used and Fourier coefficients were unweighted.

Refinement. Refinement was carried out by constrained difference Fourier methods. The two independent monomers in the asymmetric unit, designated “white” and “black,” were refined separately. A single isotropic temperature factor was used for all atoms, and contributions of solvent to the x-ray scattering were neglected. Five cycles of refinement were carried out. For each cycle, structure factors and a new difference Fourier map were calculated and shifts in each atomic position were calculated by an automated procedure (23). The coordinate shifts were projected onto the subspace of shifts that preserved bond distances and angles by use of the real-space refinement program of Diamond ("BEND" refinement with JCON = 3) (24). The atomic curvatures used in estimating shifts from the difference Fourier maps were optimized by applying

Abbreviation: Con A, concanavalin A.
arbitrary corrections based on the coefficient of correlation
between shifts obtained in successive cycles.

Following the fifth cycle of refinement, a $[2k | F_o - | F_c | - \exp(\iota \alpha_c)]$ difference map was calculated and examined for evi-
dence of changes in the structure not detected by the auto-
mated refinement. For each protomer, a model of the loop of
demetalization Con A (ref. 18 and this work) indicated that the non-
metal ligands, which comprise the 2-fold axis of the Con A tetramer move
nearly parallel to the crystallographic z axis, suggesting that the two peaks in the

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RESULTS

Initially, we made no assumptions about the arrangement of the
subunits in the apo-Con A molecule. Accordingly, we first
performed rotation functions between the apo-Con A data
and a single native Con A protomer ($M_r = 26,500$) placed in an 80
X 80 X 80 Å orthogonal unit cell in space group P1. The unit

We then initiated refinement, which proceeded smoothly, as
indicated by the decline in the R-factor and root mean square
difference density shown in Table 1. As the R-factor reached
about 0.35, the rate of improvement appeared to be decreasing,
and we decided to examine an electron density map. Residues
7-25, which are associated with metal binding, and 207-208,
which are connected by a cis peptide linkage in the native
structure, were omitted from the structure factor calculation
used to phase this map.

Best rotational and translational parameters relating the
native structure to each demetalized subunit, and the two
demetalized subunits to each other, were determined by a
least-squares minimization of the distances between corre-
sponding atoms in each pair of subunits. The final parameters
are within 1° in each rotation angle and 0.05 Å in each trans-
lation of the values originally obtained in the molecular re-
placement calculations. The noncrystallographic 2-fold axis
relating the white and black subunits of the apo-Con A dimer
is 7.37° away from the crystallographic z axis and thus from
its position in the native protein. This axis is very close to the
yz plane, resulting in nearly complete preservation of the D2
symmetry of the tetrameric molecule.

Many conformational differences are seen between the native
and demetalized structures. These changes are summarized in
Fig. 1, which gives the distances between corresponding α
carbons in the native structure and each of the two demetalized
subunits. The differences are clustered in the metal-binding
region, particularly in the loop of polypeptide chain formed
by residues 12-22. Residues 7-11 and 23-25, which were
omitted from the phases because they comprise most of the
Mn²⁺ ligands, remain in a conformation very similar to that of
the native protein. The strong appearance of these residues in
the $[2k | F_o - | F_c | - \exp(\iota \alpha_c)]$ map indicates that the phases are
quite good and suggests that regions of weaker density observed
from residues 12-22 may be due to disorder in the crystals
rather than to phasing errors.

The interpretation of residues 12-22 is difficult and not
definitive. Only those features that are common to both subunits
may be accepted with any confidence. Residues 12-22 appear
to move in such a way that the metal-binding region becomes
more accessible to solvent (Fig. 2). The SI site is little affected

Table 1. Progress of the refinement

<table>
<thead>
<tr>
<th>Stage of refinement</th>
<th>R-factor*</th>
<th>Root mean square difference density (e-/Å²)</th>
</tr>
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<tr>
<td>Starting model</td>
<td>0.4129</td>
<td>0.0865</td>
</tr>
<tr>
<td>After 1 cycle</td>
<td>0.3881</td>
<td>0.0873</td>
</tr>
<tr>
<td>After 2 cycles</td>
<td>0.3764</td>
<td>0.0839</td>
</tr>
<tr>
<td>After 3 cycles</td>
<td>0.3650</td>
<td>0.0805</td>
</tr>
<tr>
<td>After 4 cycles</td>
<td>0.3566</td>
<td>0.0777</td>
</tr>
<tr>
<td>After 5 cycles</td>
<td>0.3486</td>
<td>—</td>
</tr>
<tr>
<td>After manual</td>
<td>0.3549</td>
<td>—</td>
</tr>
<tr>
<td>corrections</td>
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</tr>
</tbody>
</table>

* R-factor = $\sum_{hkl} | |kF_o| - |kF_c| | |kF_o|$
by the changes whereas the S2 site becomes almost totally unrecognizable. Residues 12–14 appear to move in such a way that the side chain of Asn\(^{14}\) is shifted away from the S2 site in the apoprotein. The β-bend at residues 14–17, and probably the one at 15–18 as well, is apparently broken. The remaining residues connect to the β-structure strand beginning at residue 23.

In general, there are few significant conformational changes elsewhere in the molecule (Figs. 1 and 3). The changes that are observed consist mainly of rotations of Ser, Thr, and Val side chains. Among the larger residues, the side chains of Arg\(^{207}\), Arg\(^{208}\), Asn\(^{118}\), and Arg\(^{228}\) show the most notable changes. Two of these residues, Arg\(^{207}\) and Arg\(^{228}\), are in close proximity to the metal-binding region. In both subunits, the guanidinium group of Arg\(^{207}\) is disordered and occupies two positions, one near its position in the native structure and the other about 4 Å away, where it may move to avoid the altered positions of residues 17–19. In the white subunit, the guanidinium group of Arg\(^{228}\) appears to move closer to the S2 site (Fig. 2), possibly compensating in part for the missing positive charge of the Ca\(^{2+}\). Elsewhere, the rotation of Arg\(^{208}\) by about 120° about \(\chi_3\) is intriguing because of the role of this residue in stabilizing the tetrameric native structure through the formation of a salt link with Asp\(^{58}\) in another dimer (10).

An unusual feature of the native structure was the assignment of the cis configuration to the peptide linkage between Ala\(^{207}\) and Asp\(^{208}\) (9, 10). In examining the map of the apoprotein, we constructed models of residues 206–209 for both subunits, and in each case the trans configuration gave a better fit to the electron density. A cis-trans isomerization of the Ala\(^{207}\) peptide thus appears to accompany demetallization. Furthermore, Ala\(^{207}\) is more nearly in the central β-structure in both subunits of demetallized Con A than it is in the native protein (Fig. 3). Because this region of the molecule is associated with carbohydrate binding (7, 8), it is possible that the distortion of the secondary structure upon metal binding is important in the activation of the saccharide-binding site.

A number of differences between the two subunits of demetallized Con A can be seen in the electron density maps. As shown in Fig. 2, the backbone conformations in the two loop regions are different. While we can expect some details to change with further refinement, many of these differences will undoubtedly remain. Outside the loop region, the changes in Arg\(^{208}\), Asn\(^{118}\), and Arg\(^{228}\) are either different or more clearly enunciated in one subunit than in the other. The difference at Arg\(^{208}\), which is in an area of intersubunit contact, is probably correlated with the lowering of the molecular symmetry from strict 222 to nearly approximate 222 observed on the transition from native to demetallized conditions.

**DISCUSSION**

The Con A polypeptide chain can exist in a number of conformational states that are in equilibrium with each other. These equilibria are strongly modulated by the binding of metal ions and saccharides (27). For example, the presence of the metals stabilizes the saccharide-binding form, driving the equilibrium in favor of its formation. The active form is related to the demetallized form by a number of localized conformational changes, and these changes are consistent with the tendency for S1 to be occupied before S2. In the apoprotein, the transition metal binding site, S1, is mostly preformed. The protein ligands Glu\(^8\), Asp\(^{10}\), and His\(^{24}\) are in nearly the positions they have in the native structure, and Ser\(^{24}\) is in position to hydrogen bond to a water molecule that will serve as one ligand at the S1 site. On the other hand, significant shifts of Tyr\(^{12}\), Asn\(^{14}\), and Asp\(^{19}\) seem necessary to form the S2 site. It seems likely that these changes would be initiated by the binding of Asp\(^{19}\) to the metal ion at S1 and stabilized by the second metal at S2 (19). These changes would directly affect the locations of the possible saccharide-binding residues 14–16 and might influence the conformations of other residues in nearby chains that have been implicated as possible contributors to the saccharide-binding site. Residues showing such changes are located in the vicinity of Tyr\(^{100}\), Ala\(^{207}\), and Arg\(^{228}\).

The change in the configuration of the peptide bond at residue 207 is of particular interest in view of the recent suggestion, based on magnetic resonance data (27), that a cis-trans isomerization of a peptide bond may be the rate-determining step in the activation of the demetallized protein. Although the kinetic data suggest that a proline residue is involved, the location of the cis peptide at Ala\(^{207}\) in the native electron density map is unambiguous. The possibility can not yet be excluded, however, that some other proline, perhaps Pro\(^{13}\), Pro\(^{20}\), or Pro\(^{28}\), in the metal binding region, may also participate in a cis-trans isomerization. The changes in the binding region are accompanied by small changes in quaternary structure, the significance of which is not yet known. Because the demetallized crystals have been prepared at a lower pH (pH 5.0) than the native crystals (pH 6.8), the observed differences in the vicinity of the intersubunit contacts, particularly at Arg\(^{207}\), may be related more to the displacement of the dimer/tetramer equilibrium between these pH values than to any intrinsic feature of the metal-binding reaction.

The functional significance of the metal-stabilized conformational changes lies in the key role they play in the formation of the saccharide-binding form of the protein. Although a detailed analysis of these effects must await a correspondingly detailed description of the mode of saccharide binding, it is already clear that the changes induced by metals could involve many of the residues previously identified (7) as possible participants in saccharide binding.

**Note Added in Proof.** We have further refined the model of demetallized Con A described in this paper to a present R-factor of 0.3349. Although the apparent disorder in residues 12–22 may preclude a precise description of their structure, the disorder in itself would constitute a significant difference from the native structure, in which these residues are well-ordered, and would correlate with the decreased ability of the demetallized protein to bond Ca\(^{2+}\) and saccharides.
FIG. 2. Stereoscopic drawings superimposing the metal-binding regions in native Con A and the white subunit (a) and native Con A and the black subunit (b) of demetallized Con A. The molecules are viewed down the z axis, with 110 vertical. The demetallized subunits have been aligned for maximum overlap with the native protein. Heavy atoms and solid bonds are used for the demetallized structure and light atoms and open bonds for the native. Peptide groups are represented by a single bond between α carbons, except at residues 12, 32, and 228, where the entire peptide group is shown. Labels indicate residues that play an important role in metal binding (19), for which the side chain or peptide group, as appropriate, is shown.

FIG. 3. Superposition of the entire α carbon backbone of native Con A and the demetallized black subunit. Orientation and symbols are as in Fig. 2. Deviations from perfect alignment are largest in the region of metal- and saccharide-binding.
We thank Drs. R. A. Crowther, R. Diamond, and C. K. Johnson for some of the computer programs used in this study. We also thank K. Jones-Mulry for excellent technical assistance and Mr. S. Altschul for help with computer programming. G. N. R. was the recipient of an Alfred P. Sloan Research Fellowship. This research was supported by National Institutes of Health Grant AI-11378, and development of computer programs was supported by National Institutes of Health Grant GM-22663.