Cadmium-Substituted Concanavalin A and Its Trimeric Complexation

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Concanavalin A (ConA) interacts with carbohydrates as a lectin, and recent reports proposed its application for detecting a diversity of viruses and pathogens. Structural studies have detailed the interaction between ConA and carbohydrates and the metal coordination environment with manganese and calcium ions (Mn-Ca-ConA). In this study, ConA was crystallized with a cadmium-containing precipitant, and the refined structure indicates that Mn^{2+} was replaced by Cd^{2+} (Cd-Ca-ConA). The structural comparison with ConA demonstrates that the metal-coordinated residues of Cd-Ca-ConA, that is Glu8, Asp10, Asn14, Asp19, and His24, do not have conformational shifts, but residues for sugar binding, including Arg228, Tyr100, and Leu99, reorient their side chains, slightly. Previous studies demonstrated that excess cadmium ions can coordinate with other residues, including Glu87 and Glu183, which were not coordinated with Cd^{2+} in this study. The trimeric ConA in this study coordinated Cd^{2+} with other residues, including Asp80 and Asp82, for complex generation. The monomer does not have specific interaction near interface regions with the other monomer, but secondary cadmium coordinated with two aspartates (Asp80 and Asp82) from monomer 1 and one aspartate (Asp16) from monomer 2. This study demonstrated that complex generation was induced via coordination with secondary Cd^{2+} and showed the application potential regarding the design of complex formation for specific interactions with target saccharides.

Keywords: Biotechnology of lectins, concanavalin A, cadmium substitution, metal coordination

Introduction

Extensive functional investigations of ConA have been performed regarding the importance of its sugar-binding abilities, with the results providing crucial information to the field of protein-protein interactions [1, 2]. The reported ConA structures have bivalent metal ions: Mn^{2+} and Ca^{2+} . The S1 site, which is the Mn^{2+} -coordinated region, can be easily replaced with other metal ions, such as zinc, cadmium, calcium, and cobalt, as compared to the S2 site, which is the Ca^{2+} -coordinated region [3, 4]. The use of amino acids for sugar interactions through hydrogen bonds and ionic interactions already has been shown, with these residues modified to achieve sufficient binding based on the substrates. These residues usually consist of carboxylic

groups in ConA and generate hydrogen bonds with hydroxyl moieties in carbohydrates. Extensively studied ConA species, including *Canavalia gladiata* and *Canavalia ensiformis*, have high sequence identity, and biophysical assays indicate that they have nearly identical binding affinities to norovirus GII.4 genotypes [2, 5, 6]. In addition, mutational studies illustrated that the sugar-binding sites are not crucial for the interaction with norovirus; rather, it was influenced by bivalent metal coordination residues, including Glu8, Asp10, Asn14, Asp19, and His24, in ConA. These results were confirmed through hydrogen/deuterium exchange mass spectrometry (HDX-MS), and interaction details were investigated through patterns of complex formation from ConA [2]. The reported structures of ConA were usually dimers or tetramers, but a recent update of

the monomeric structure at 1.6 Å resolution demonstrated that a specific amino acid was oriented differently based on the complex formation [1]. This difference could be a critical issue for detecting and determining the binding partners of ConA, especially concerning the development of detection and/or concentration determination kits consisting of protein-based beads. Although ConA contains the same bivalent metal ions, the side and main chains of sugar-binding residues including Tyr12, Asn14, Leu99, Tyr100, Asp208, and Arg228 can have different orientations, such as, dimer, trimer, and tetramer, on the basis of the complex formation.

Ions of the toxic metal cadmium were detected in the manganese-binding site during the structural studies of ConA owing to the presence of this metal in the screening precipitant employed in this study. The structural features of this Cd²⁺ and Ca²⁺-coordinated ConA (Cd-Ca-ConA) were found to be different from those of Mn²⁺ and Ca²⁺-coordinated ConA (Mn-Ca-ConA) and monomeric Cd-Ca-ConA because the crystallization conditions are different [1, 7–9]. Metal-coordinated regions, sugar-binding environments, and loop regions near sugar-binding residues including Ser201, Pro202, Ser204, and His205 were investigated and conformation changes were explained. Reported results indicated that Co²⁺, Ni²⁺, and Zn²⁺ can replace Mn²⁺, although Cd²⁺ can coordinate with S1 and S2 sites [4, 10–12]. The titration studies also found that Cd²⁺ can generate a third-metal coordination site, S3. Trimeric Cd-Ca-ConA generates a specific Cd²⁺-coordinated S3 site, which uses different amino acids at other locations [9]. In addition, this unique coordination with cadmium at S3 leads to chelation with the other monomer. With this study, possible changes in side chains based on the hetero-metal coordination in ConA can be proposed, which would guide protein engineering in the effort to generate specific protein-protein interactions.

Materials and Methods

Protein Crystallization and Data Collection

ConA (from *Canavalia ensiformis*; Sigma, C7275) was crystallized with 0.1 M cadmium chloride hydrate, 0.1 M sodium acetate trihydrate (pH 4.6), and 30% v/v polyethylene glycol 400. A rhombic crystal was frozen by a solution consisting of 10% glycerol to avoid X-ray damage and then was mounted on a 0.2 mm cryo-loop for data collection. The X-ray diffraction data sets of the crystal were obtained using an ADSC Quantum 270 CCD detector on the beamline BL-7A of the Pohang Accelerator (PLS), Republic of Korea, at a wavelength of 1.000 Å.

Table 1. Data collection and refinement statistics for Cd-Ca-Con from *Canavalia ensiformis*.

Structure	ConA trimer ^a (PDB code: 6AHG)
Space group	I422
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	<i>a</i> = 136.46 Å, <i>b</i> = 136.46 Å, <i>c</i> = 193.15 Å
α , β , γ (°)	$\alpha = \beta = \gamma = 90^\circ$
Resolution (Å)	48.29 – 2.83 (2.98 – 2.83) ^b
R_{merge}^c	0.098 (0.457)
$I/\sigma I$	15.5 (4.8)
Completeness (%)	99.9 (99.5)
Redundancy	7.2 (7.3)
Resolution (Å)	48.29 – 2.83
No. reflections	21046
$R_{\text{work}}/R_{\text{free}}$ (%)	24.32/27.92
No. of non-H atoms/ average B factor (Å ²)	5404/49.52
Protein	5376
Ca ²⁺	3
Cd ²⁺	6
Water	19
Average B factor (Å ²)	49.52
Wilson B factor (Å ²)	56.70
R.m.s. deviations	
Bond lengths (Å)	0.009
Bond angles (°)	1.634
Ramachandran plot (%) ^d	
Most favored	97.4
Allowed	2.6
Disallowed	0

^aOne crystal was used.

^bValues in parentheses are for the highest-resolution shell.

^c $R_{\text{merge}} = \sum_{hkl} \sum_j |I_{hkl}(j) - \langle I_{hkl} \rangle| / \sum_{hkl} \sum_j I_{hkl}(j)$ where $\langle I_{hkl} \rangle$ is the mean intensity and $I_{hkl}(j)$ are individual intensity measurements of the reflection (*hkl*).

^dAs defined in the program PROCHECK (Laskowski, 1998).

Structure Determination

The 2.83 Å data were indexed, integrated, and scaled using the XDS software package [13]. The processing statistics for Cd²⁺-Ca²⁺-ConA are listed in Table 1. Data analysis of the crystal showed that it belonged to space group I422, with *a* = 136.46 Å, *b* = 136.46 Å, *c* = 193.15 Å, and $\alpha = \beta = \gamma = 90^\circ$. The asymmetric unit contains three ConA molecules with a solvent content of 56.26% by volume per protein weight, V_M of 2.81 Å³ Da⁻¹ [14]. The crystal structure of ConA from *Canavalia ensiformis* (PDB code: 5YGM) was used as a

template model for the molecular replacement method (Collaborative Computational Project, [1]). To determine the structural solution of ConA, some trials of molecular replacement were performed using PHASER [15]. Further model building and refinement of ConA was performed with WinCoot and REFMAC5.8 in CCP4, respectively [16]. The R values of the final model were R_{work} and R_{free} of 24.32% and 27.92%, respectively. Thus, the final structure was validated by PROCHECK [17].

Results and Discussion

Structure of Trimeric Cd^{2+} and Ca^{2+} ConA from X-Ray Crystallography

It was considered that Mn^{2+} in metal-coordinated residues does not affect the binding affinity for saccharides, because detailed interactions were determined from the sugar-binding residues near Ca^{2+} -coordinated amino acids, including Tyr and Arg residues, generating major hydrogen bonds [10, 18–21]. During the crystallization of ConA in this study, the precipitant for crystallization contained 100 mM cadmium chloride hydrate, a xenobiotic metal ion that replaced the Mn^{2+} -coordinated site. The Ca^{2+} -coordinated region was not substituted in the ConA crystal study; it was refined to 2.83 Å resolution (Fig. 1, Table 1, and PDB accession: 6AHG). Structures of ConA from *Canavalia ensiformis* were reported from more than 58 different forms, and two studies explained cadmium-substituted ConA. It was reported that Cd-Cd-ConA crystal can be obtained from apo-ConA through demetallization [10]. The other deposited structure was a monomeric Cd-Ca-ConA with a secondary Cd^{2+} -coordinated site located opposite the metal-coordinated region [9]. The

reported trimeric Cd-Ca-ConA with secondary Cd-ion-coordinated structure resembles the latter case, which implies that Ca coordination to ConA is not easily replaced with other metal ions.

Three monomers generate trimeric Cd-Ca-ConA, each of which shows high structural similarity (Figs. 1A and 1B). These results were confirmed by 0.059–0.062 root-mean-square (RMS) values through $\text{C}\alpha$ alignment of each monomer. Overall shape and interactions among the monomers showed identical patterns. The interactions between monomers do not have a specific hydrogen bond or hydrophobic interaction, but the secondary Cd ion is a generator of interactions with the other monomers. The coordination environment of the secondary Cd ion is quite different compared to the previously reported secondary Cd ion in Cd-Ca-ConA (PDB: 1CON, *vide infra*). The core region of the ConA structure is rigid owing to two anti-parallel β -sheets, but metal-coordinated regions are mostly positioned in loops and short α -helices [7, 22]. The secondary Cd^{2+} also was positioned in these flexible regions and it can lead to complex formation [9].

Superimposed Structures of Mn-Ca-ConA and Cd-Ca-ConA

Metal ion replacement was more favorable in the S1 than in the S2 site. The superimposed structure of bivalently (Mn^{2+} and Ca^{2+}) coordinated ConA (Mn-Ca-ConA, PDB accession: 2CTV, [9]) and cadmium-substituted ConA (Cd-Ca-ConA, reported as ConA in this study) has high structural similarity in overall shape (RMS = 0.420), including metal-coordinated regions and the core region

Fig. 1. Structure of trimeric ConA (PDB accession: 6AHG).

(A) Ribbon diagram of trimeric Cd-Ca-ConA. Orange balls represent calcium ions, and light-blue balls represent cadmium ions. (B) Surface model of trimeric Cd-Ca-ConA.

Fig. 2. Structural comparison between ConA (Mn-Ca-ConA; PDB accession: 2CTV) and trimeric Cd-Ca-ConA (PDB accession: 6AHG).

(A) Overall superimposed structures between Mn-Ca-ConA (pale orange) and Cd-Ca-ConA (dark green) and (B) the metal-coordinated region show high structural similarity (RMS = 0.420). Red and blue represent atoms of oxygen and nitrogen, respectively. Orange and cyan balls represent calcium ions from ConA and Cd-Ca-ConA, respectively. Blue and light-blue balls represent Mn and Cd ions, respectively.

consisting of two β -sheets (Fig. 2A). Each β -sheet has six and seven β -strands comprising the main scaffold of ConA, and these strands are connected with loops. The bivalent ions are coordinated with residues in these loops, and sugar-binding residues are positioned in the loops between β -strands. The structural comparison established that Cd^{2+} - or Mn^{2+} -coordinated residues, including Glu8, Asp10, Asn14, Asp19, and His24, coordinated with bivalent ions in almost identical manners, as shown in Fig. 2B.

The metal coordination region is considered an important functional moiety for interactions with its binding partners,

and side-chain positions of these residues affect carbohydrate-coordinated regions. Monomeric ConA (Mn-Ca-ConA) and apo-ConA have different orientations of side chains, including Glu8 and Asp14, and these variations further induce positional changes in the sugar-binding region [1, 11, 18]. Apo-ConA changes the location of sugar-binding residues, including Tyr12, Asn14, Leu99, Tyr100, Asp208, and Arg228, which are located in the loop region near the metal-coordinated region. These results were confirmed from mutational studies of the metal-coordinated region and measurements of binding affinities [2]. The superimposed structure of monomer and dimer ConA shows identical Mn^{2+} and Ca^{2+} coordination, but their sugar-binding residues have slightly changed orientations. Owing to these flexibilities of the sugar-binding region located mostly in loops between β -strands, its binding abilities in lectins need to be investigated at the atomic level.

Conformational Changes in the Sugar-Binding Region

Although metal coordination residues are identical, the superimposed information between Cd-Ca-ConA and Mn-Ca-ConA has crucial sugar-binding residues. Residues including Arg228, Leu99, and Tyr100 show slightly shifted positions on the basis of metal or complex generation although the limited resolution of Cd-Ca-ConA cannot confirm further details. Arg228 of ConA forms hydrogen bonds with hydroxyl groups of monosaccharides, disaccharides, and trisaccharides, but Cd^{2+} -substituted ConA affects the position of the side chain of Arg228 (Fig. 3A) and this event may influence interactions between ConA and its binding partners [3, 4, 7, 8, 10, 22]. While the amide moiety of the side chain of Arg228 shows

Fig. 3. Structural comparison of ConA (PDB accession: 2CTV) and trimeric Cd-Ca-ConA (PDB accession: 6AHG) sugar-binding residues.

(A) Side-chains from Arg228, Leu99, and Tyr100 show slight changes after Cd replacement. Orange and cyan balls represent calcium ions from ConA and Cd-Ca-ConA, respectively. Blue and light-blue balls represent Mn and Cd ions, respectively. (B) The secondary sugar-binding region consists of Ser201, Pro202, Ser204, and His205. Red and blue represent atoms of oxygen and nitrogen, respectively.

conformational change by the Cd substitution, Tyr100 shows a slight shift owing to the small shifts in the loop position from Thr97 to Thr103. Among these sugar-binding residues, Leu99 forms hydrogen bonds through the main chain with the hydroxyl moiety of carbohydrates. The conformational change in the side chain of Leu99 is not significant but rotation was induced by the shift of Tyr100 (Fig. 3A).

Previous reports proposed that the secondary carbohydrate-binding region near carbohydrate interaction residues is influenced based on the assemblies of ConA [1, 4], but the structural information illustrated that the coordination of these residues is not changed by the cadmium ions in this study (Fig. 3B). These residues, including Ser201, Pro202, Ser204, and His205, are positioned near sugar-binding residues, and positions of side chains were not influenced directly by their binding partners. Apo-ConA has changed coordination in metal-coordinated residues and sugar-binding regions [10, 11]. While the ConA dimer has identical positions of side chains in metal coordination, its secondary sugar-binding residues show a different conformation of these side chains. The positions of carbohydrate-binding residues can be moved depending on the metal-coordinated residues and first sphere of carbohydrate interaction residues.

Secondary Cadmium Ion Triggers Trimeric Cd-Ca-ConA

The determined binding affinities of cadmium ions in biological systems have a lower dissociation constant (K_d) as compared to those of other metal ions, and reports demonstrate that this hazardous metal ion can replace physiological metal ions of ConA and other proteins [6, 23, 24]. Monomeric Cd-Ca-ConA was determined through X-ray crystallography (PDB accession: 1CON). Crystallization in the current study shows a Cd-Ca-ConA trimer per unit cell. The positions of side chains from metal-coordinated residues and carbohydrate-binding residues are identical in both structures, although their assemblies are different, *i.e.*, monomer vs. trimer [9]. Both structures have another metal-binding site (S3), and these coordinates with the second cadmium require different amino acids. The superimposed structure shows high similarity, with 0.407 RMS. Trimeric Cd-Ca-ConA coordinates with aspartate residues (Asp80 and 82 from monomer 1 and Asp16 from monomer 2), but monomeric Cd-Ca-ConA uses glutamate residues (Glu87 and Glu183) for the second cadmium coordination, as shown in Fig. 4.

The second cadmium ion of monomer 1 from trimeric

Fig. 4. Superimpositions of monomeric ConA (Cd-Ca-ConA monomer; PDB accession: 1CON) and trimeric Cd-Ca-ConA (PDB accession: 6AHG).

(A) Monomeric and trimeric structure of Cd-Ca-ConA has structural similarity (RMS = 0.407), but secondary Cd coordinated to different amino acids in ConA (box). Orange and yellow balls represent calcium ions from trimeric Cd-Ca-ConA and monomeric Cd-Ca-ConA, respectively. (B) The secondary Cd binds to different regions of ConA. Light-blue and green balls represent secondary Cd ions from trimeric and monomeric ConA, respectively.

Cd-Ca-ConA coordinates with an aspartate residue (Asp16) from monomer 2 (Fig. 5A), but the monomeric structure does not coordinate with another monomer at the S3 site. Asp16 coordinates with the second Cd^{2+} , and this residue does not overlap with other residues in metal-coordinated or sugar interactions. The conformational changes of Asp16 for the coordination with Cd^{2+} in another monomer requires slight rotation, which was monitored, compared to the monomer Cd-Ca-ConA (PDB accession: 1CON), as shown in Fig. 5B [10]. Slight rotation is easily allowed in trimer ConA, because the Asp16 was placed in a short α -helix connected with loops. The reported structure of Cd-Ca-ConA indicated that the second cadmium coordinates with Glu87 and Glu183 and distorted octahedral geometry was completed with Asp16 from another monomer and two water molecules. The trimeric structure of Cd-Ca-ConA also coordinated with three aspartate residues and two water molecules, although resolution was limited for further confirmation (Fig. 5C).

Results from calorimetric titration studies illustrated that cadmium ions influence complex formation [4]. This was determined by the presence of a high temperature transition peak (T2). Two monomers enhance interaction energy through coordination with metal ions and with carboxylase moieties, as the first monomer has Glu87 and Glu183 and the second monomer coordinates Cd^{2+} with Asp16. These results were considered as the main factors causing the T2

Fig. 5. Secondary cadmium ion generates trimeric ConA.

(A) The secondary Cd ion coordinated with Asp80 and Asp82 from monomer 1 (dark green) and Asp16 from monomer 2 (purple). (B) Reorientation of Asp16 for the complex formation is detected from superimposed monomeric and trimeric structures. Purple and dark green represent monomer 1 and monomer 2, respectively, from trimeric Cd-Ca-ConA. Yellow represents monomeric Cd-Ca-ConA. Light-blue balls represent cadmium ions, and red represents oxygen atoms. (C) Electron density map of Ca²⁺- and Cd²⁺-coordinating residues shown by 2Fo-Fc electron density map (black) contoured at 1 sigma (σ).

transition peak during ITC (isothermal titration calorimetry) and DSC (differential scanning calorimetry) titration. The trimeric complex in this study has different coordination for the second Cd²⁺-binding site at quite different positions of ConA, but it generates quite similar coordination in trimeric ConA. This is one of the major binding affinities between monomers. Dimer generation shows different patterns, because the major interactions were generated through β -sheets of each monomer. These were confirmed by the Zn-Ca-ConA dimer (PDB accession: 3ENR), as the major interactions were monitored from β -sheets [10]. Although this dimer has a second zinc coordination site, S3, the complex is not a trimer and this ion does not participate in any complex formation. This result indicated that complex formation of ConA can induce changed orientations of side chains from metal-coordinated residues and sugar-binding residues.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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