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Quaternary structure of *Dioclea grandiflora* lectin assessed by equilibrium sedimentation and crystallographic analysis of recombinant mutants

Sara Zamora-Caballero [a], Alicia Pérez [b], Libia Sanz [b], Jerónimo Bravo [a,*], Juan J. Calvete [b,*]

[a] *Unidad de Transducción de Señales, Instituto de Biomedicina de Valencia, CSIC, Spain*
[b] *Laboratorio de Venómica Estructural y Funcional, Instituto de Biomedicina de Valencia, CSIC, Spain*

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ABSTRACT

The structural basis of the pH dependency of the dimer–tetramer transition exhibited by Brinda's type II *Diocleinae* lectins was investigated by equilibrium sedimentation and X-ray crystal structure determination of recombinant wild-type and site-directed single and double mutants of the pH-stable tetrameric *Dioclea grandiflora* lectin (r-αDGL). Releasing the peripheral site interdimeric contact between R60 and D78 rendered a mutant displaying dimer–tetramer equilibrium in the pH range equivalent to pKa ± 1 of the γ-COOH. Mutation of both histidines 51 and 131, but not the mutation of each His separately, abolished the formation of the Diocleinae canonical tetramer in the pH range 2.5–8.5. The X-ray structure of the double mutant r-αDGL H51G/H131N suggests that H131 plays a crucial role in networking loop 114–125 residues from all four subunits at the central cavity of the tetrameric lectin, and that H51 maintains the central cavity loops in a proper spatial orientation to make H131-mediated interdimer contacts.

1. Introduction

Ubiquitous in all the kingdoms of life, lectins are proteins of non-immune origin, that recognize specific sugar structures attached to soluble and integral cell membrane glycoconjugates [12]. Compared to proteins and nucleic acids, carbohydrates are unbeatable in information-coding potential. Due to their ability to recognize specific glycocodes, lectins play pivotal roles in carbohydrate-mediated biological processes, such as communication between cells, tumor metastasis, innate immunity, sperm–egg recognition, infections by parasites, plant protection against phytopathogens and phytopredators, etc. [11]. Lectins are not only valuable research tools for the molecular understanding of the sugar code [13,2] but also attractive molecules for targeting drugs to specific destinations [18].

Lectins isolated from the seeds of genera *Dioclea*, *Cratylia*, and *Canavalia* of the *Diocleinae* subtribe of the *Papilionoideae* subfamily of *Leguminosae* display the back-to-back arrangement of two dimers built by the side-by-side, antiparallel alignment of the subunit six-stranded back β-sheets [20], first reported in the X-ray structure of *Canavalia ensiformis*' seed lectin, concanavalin A [25]. Type II legume lectin tetramers (*sensu* [6] exhibit pH-dependent dimer–tetramer equilibrium [17,28,29]. A $25 \times 8\ \text{Å}^2$ water-filled central cavity formed upon tetramerization has been reported to bind non-polar molecules, such as plant cytokinins and β-indoleacetic acid [8,26]. These molecules act as plant growth hormones and thus *Diocleinae* lectin dimer–tetramer transition could serve as a cargo mechanism for these hormones between different parts of the plant. Reported pH values in the xylem–lumen space of higher plants range from 3.5 to 8.3, depending on plant species, plant parts, experimental conditions and method used [33], and the pH values of the two main cellular compartments, the cytoplasm and the vacuole, are, respectively, slightly alkaline (7.4–7.5) and markedly acid (4.5–6.0) [16]. However, the precise distribution of the physiological pH in the different tissues, cells, subcellular compartments, and fluids of the plant is needed to support the "cargo hypothesis". Nonetheless, knowledge of the structural determinants that modulate the quaternary assembly of *Diocleinae* lectins would facilitate the long-sought biotechnological

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Author contributions: JJC and LS conceived and supervised the study and designed experiments; AP and LS produced the recombinant wild-type and site-directed lectins; AP performed crystallization experiments; JB and SZ-C collected and analyzed the crystallographic data; JJC, JB and SZ-C wrote the manuscript; all authors made manuscript revisions.

* Corresponding authors at: Instituto de Biomedicina de Valencia, CSIC, Jaime Roig 11, 46010 Valencia, Spain. Fax: +34 96 369 0800.

E-mail addresses: jbravo@ibv.csic.es (J. Bravo), jcalvete@ibv.csic.es (J.J. Calvete).

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use of these lectins as transport proteins to release its cargo to their specific (natural or engineered) glycan ligand-bearing destinations (Vasta and Ahmed [31]).

Structural comparisons of the crystal structures of pH-independent tetrameric lectins (*D. grandiflora* (1DGL, [27], and *Dioclea violacea* (2GDF, [14]; 3AX4, [4] lectins) and homologous lectins that exhibit pH-dependent dimer–tetramer transitions, *D. guianensis* lectin (DguiaL) (1H9P, 1H9 W; [32] and *Cratylia floribunda* lectin (CFL, 2D3R, 2D3P; [15], have revealed that the dimer–tetramer equilibrium depends on interdimeric contacts between regions involving homologous residues 53–78 of opposing monomers (A and D and B and C) at the periphery of the dimers, and the loops formed by residues 117–123 of each monomer facing the central cavity of the tetramer (Fig. 1). More recently, the development of a system for the expression of recombinant DGL (P08902) and DguiaL (A9J248), structurally and functionally indistinguishable from their natural versions [23], has opened the door to a more detailed analysis of the interactions that govern the tetrameric association of *Diocleinae* lectins. Thus, conversion of the pH-stable tetrameric r-DGL into a structure exhibiting pH-dependent dimer–tetramer transition was accomplished through three simultaneous mutations, E123A/H131N/K132/Q, that abolished the interdimeric contacts networking the central cavity loops [23]. Here, we report the distinct structural impact of single and double mutations affecting residues H51, R60, and H131, assessed by equilibrium sedimentation and crystallographic analysis.

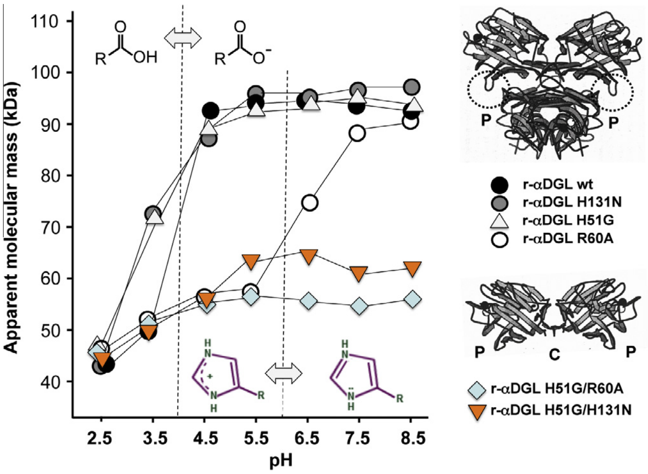

Fig. 1. Determination by equilibrium sedimentation analytical centrifugation of the apparent molecular masses of the recombinant lectins as a function of the pH of the solution. Apparent molecular masses (M_{app}) between a pure dimer (*D*, 51kDa) and a pure tetramer (*T*, 102kDa) correspond to $M_{app} = (\%D \times 51 + \%T \times 102)$ kDa. The location of the peripheral (P) and central (C) interdimeric regions are indicated in the structures of the canonical dimer and tetramer of r-α-DGL [1DGL, 2JDZ]. Vertical broken lines indicate the pKa3 (3.9) of aspartic acid (β-COOH) and the pKa (6.0) of the imidazole group of histidine.

2. Materials and methods

2.1. Generation, expression, and purification of recombinant lectins

Construction of a synthetic gene encoding the wild-type full-length α-chain of *D. grandiflora* lectin [P08902] (r-α-DGL) was performed as described previously [23]. Generation of single (H51A, H51G, R60A) and double (H51A/R60A, H51G-H131N) mutants was done using the QuickChange® site-directed mutagenesis kit (Stratagene) as described [23]. Briefly, pET32a plasmids containing the template sequences (Table 1) flanked by NcoI and NotI restriction sites were PCR-amplified [initial denaturation at 94 °C for 2 min, followed by 16 cycles of denaturation (30 s at 94 °C), annealing (60 s at 55 °C), and extension (13 min at 68 °C), and a final extension for 10 min at 68 °C] using the forward (F) and reverse (R) primers listed in Table 1, designed for generating the site-directed mutants. Amplified products were sequenced (using an Applied Biosystems model 377 DNA sequencer) to confirm the correctness of the mutants.

For expression of recombinant wild-type and mutated r-α-DGL, *Escherichia coli* BL21(DE3) cells (Novagen) were transformed by electroporation with the plasmid DNAs. Positive *E. coli* BL21 (DE3) clones, shown by PCR to contain the wild-type and the mutated r-α-chain-thioredoxin-His$_6$ fusion constructs, were grown overnight at 37 °C in LB medium containing 100 μg/mL of ampicillin. For inducing the expression of the recombinant fusion proteins, the cell cultures were diluted 1:10 (v/v) with medium, isopropyl-β-D-thiogalactosidase (IPTG) was added to a final concentration of 1 mM, and the cell suspensions were incubated for another 24 h at 14 °C. Thereafter, the cells were pelleted by centrifugation, resuspended in the same volume of 20 mM sodium phosphate, 150 mM NaCl, pH 7.4, washed three times with the same buffer, and resuspended in 100 mL/L of initial cell culture of 20 mM sodium phosphate, 250 mM NaCl, 10 mM imidazole, pH 7.4. The cells were lysed by sonication (15 cycles of 15 s sonication followed by 1 min resting) in an ice bath. The lysates were centrifuged at 10000×g for 30 min at 4 °C, and the soluble and the insoluble fractions were analyzed by SDS–15% polyacrylamide gel electrophoresis.

The α-chain-thioredoxin-His$_6$ fusion wild-type and mutated r-DGL proteins were purified from the soluble fraction of positive *E. coli* BL21 (DE3) clones by affinity chromatography using an ÄKTA Basic chromatographer equipped with a 5-mL HisTrap HP column (Amersham Biosciences) equilibrated in 20 mM sodium phosphate, 250 mM NaCl, 10 mM imidazole, pH 7.4. After the absorbance at 280 nm of the flowthrough fraction reached

Table 1
Forward (F) and reverse (R) primers used for generating the site-directed mutants of the α-chains of the seed lectins of *Dioclea grandiflora* (r-α-DGL). Codons producing the mutations are in boldface and underlined.

Mutant	Template used	Primers (5′–3′)
r-α-DGL H131N	r-α-DGL wt	F: TCA CTC CAT TTC AGC TTC **AAC** AAA TTT AGC CAA AAC CCA AAG G R: GG GTT TTG GCT AAA TTT **GTT** GAA GCT GAA ATG GAG TGA ATT TT
r-α-DGL H51G	r-α-DGL wt	F: GGG AAG GTA GGA ACT GTT **GGC** ATA AGC TAC AAC TCT GTC GC R: GC GAC AGA GTT GTA GCT TAT **GCC** AAC AGT TCC TAC CTT CCC
r-α-DGL R60A	r-α-DGL wt	F: GC TAC AAC TCT GTC GCT AAG **GCA** CTA AGT GCT GTT GTT TCT TAT TCT GG R: CC AGA ATA AGA AAC AAC AGC ACT TAG **TGC** CTT AGC GAC AGA GTT GTA GC
r-α-DGL H51G/R60A	r-α-DGL H51G	F: GC TAC AAC TCT GTC GCT AAG **GCA** CTA AGT GCT GTT GTT TCT TAT TCT GG R: CC AGA ATA AGA AAC AAC AGC ACT TAG **TGC** CTT AGC GAC AGA GTT GTA GC
r-α-DGL H51G/H131N	r-α-DGL H131N	F: GGG AAG GTA GGA ACT GTT **GGC** ATA AGC TAC AAC TCT GTC GC R: GC GAC AGA GTT GTA GCT TAT **GCC** AAC AGT TCC TAC CTT CCC