The mannose-specific lectin domains of Flo1p from *Saccharomyces cerevisiae* and Lg-Flo1p from *S. pastorianus*: crystallization and preliminary X-ray diffraction analysis of the adhesin–carbohydrate complexes

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Flo1p and Lg-Flo1p are two cell-wall adhesins belonging to the Flo (flocculation) protein family from the yeasts *Saccharomyces cerevisiae* and *S. pastorianus*. The main function of these modular proteins endowed with calcium-dependent lectin activity is to mediate cell–cell adhesion events during yeast flocculation, a process which is well known at the cellular level but still not fully characterized from a molecular perspective. Recently, structural features of the N-terminal Flo lectin domains, including the N-terminal domain of Lg-Flo1p (N-Lg-Flo1p), and their interactions with carbohydrate molecules have been investigated. However, structural data concerning the N-terminal domain of Flo1p (N-Flo1p), which is the most specific among the Flo proteins, are missing and information about the N-Lg-Flo1p–carbohydrate interaction still lacks detailed structural insight. Here, the crystallization and preliminary X-ray characterization of the apo form and the mannose complex of N-Flo1p and X-ray analysis of N-Lg-Flo1p crystals soaked in α -1,2-mannobiose are reported. The N-Flo1p crystals diffracted to a resolution of 1.43 Å in the case of the apo form and to 2.12 Å resolution for the mannose complex. Both crystals were orthorhombic and belonged to space group $P2_12_12_1$, with one molecule in the asymmetric unit. The N-Lg-Flo1p– α -1,2-mannobiose complex crystal diffracted to 1.73 Å resolution and belonged to the monoclinic space group $P12_11$ with two molecules in the asymmetric unit.

1. Introduction

Cellular adhesion is a key process in yeast behaviour. It can be used for ‘offensive’ purposes in the case of human pathogenic *Candida* yeasts, where it is the first step in host-cell colonization and invasion. It can also represent a ‘defensive’ system, for example when *Saccharomyces* spp. self-aggregate, form flocs and can escape from harsh environmental conditions by sedimentation of the flocs. No matter from which perspective this process is looked at, it has the univocal meaning of allowing yeast survival in different biological contexts.

‘Flocculation’ is the common term used to indicate self-adhesion and aggregation of yeast cells. This phenomenon is very familiar to brewers as a simple and economic system to separate yeast cells from beer at the end of the primary fermentation. Yeast flocculation has been extensively investigated during the last half century using the model yeast *Saccharomyces cerevisiae* (Verstrepen & Klis, 2006; Goossens & Willaert, 2010; Brückner & Mösch, 2012). The formation of yeast flocs generally takes place under starvation conditions (*i.e.* in the absence of fermentable carbohydrates in the yeast environment) and is calcium dependent. Moreover, it is mainly a carbohydrate-sensitive reversible process. This key feature is linked to the expression of different Flo (flocculation) adhesins or flocculins, a family of glycosylphosphatidylinositol-anchored cell-wall proteins (GPI-CWPs; Dranginis *et al.*, 2007; Van Mulders *et al.*, 2009). Structures of Flo1p, Flo5p, Flo9p and Flo10p, which are responsible for the carbohydrate-sensitive *S. cerevisiae* flocculation, are organized in

three domains, namely an N-terminal adhesive domain, a central Ser/Thr-rich domain (for the extension of the adhesive domain outwards from the cell wall) and a C-terminal domain, which is linked to the GPI anchor.

The N-terminal domain is characterized by C-type lectin activity and it confers a Ca^{2+} -mediated carbohydrate specificity to each Flo protein (Kobayashi *et al.*, 1998). This specificity can be broad or narrow, depending on which carbohydrate molecules can abolish cell–cell adhesion by blocking the interaction of the lectin domain with the cell-wall glycans. The yeast flocculation phenotype may belong to the ‘Flo1’ type, when the flocculation phenotype is only mannose sensitive, or to the ‘Newflo’ type, when the flocculation can be inhibited by mannose and other sugars such as glucose or saccharose (Stratford & Assinder, 1991). While Flo1p possesses the narrowest specificity, Flo10p is a representative of the ‘Newflo’ group (Van Mulders *et al.*, 2009).

Lg-Flo1p also belongs to the Flo family. It was first discovered in the lager yeast *S. pastorianus* (Kobayashi *et al.*, 1998), but has been found recently also in different ale *S. cerevisiae* strains (Van Mulders *et al.*, 2010). It is a Flo1p homologue and it is able to confer a ‘Newflo’ flocculation phenotype, since it is able to interact not only with mannose but also with other sugars such as glucose or maltose.

Recently, three-dimensional structures of the N-terminal domains of Flo5 and Lg-Flo1 have been solved by X-ray crystallography. An in-depth structural and functional analysis of the interaction of N-Flo5 with mannose and mannosides has been carried out (Veelders *et al.*, 2010). It revealed a prevalent β -sandwich folding with an unusual calcium-binding *cis*-peptide motif and a high specificity of the Flo5 flocculin for α -1,2-mannobiose, which is a disaccharide moiety commonly found in cell-wall mannans. This study also confirmed that the Flop adhesive domains belong to the PA14 family (Rigden *et al.*, 2004), a group of proteins that show homology to a 14 kDa fragment of the anthrax toxin protective antigen (PA) and also includes the N-Epa1p lectin from the pathogenic yeast *Candida glabrata* (Ielasi *et al.*, 2012). Recently, it was shown that the N-terminal domain of Lg-Flo1p has several traits in common with the N-Flo5p structure (Sim *et al.*, 2013). The affinity of this domain for a large set of carbohydrates was determined, but a detailed crystallographic characterization of the protein in complex with a carbohydrate ligand was lacking.

In this work, we describe the crystallization and preliminary X-ray diffraction analysis of the N-Flo1p and N-Lg-Flo1p lectin domains. N-Flo1p was easily crystallized in different conditions and was eventually analysed in the form of a ligand-free crystal and as a crystal soaked in a mannose ligand solution. On the other hand, N-Lg-Flo1 crystallization was more difficult to achieve. The obtained crystals were soaked in a α -1,2-mannobiose ligand solution to analyse the interaction with this carbohydrate.

2. Materials and methods

2.1. Protein expression and purification

The *flo1* gene (UniProt entry P32768) from *S. cerevisiae* (BY4742), spanning residues 27–271, and the *lg-flo1* gene (UniProt entry B3IUB3) from *S. pastorianus* (CBS1513), spanning residues 23–247, were separately cloned into the pET-21b(+) expression vector (EMD Chemicals) by using the *NdeI/XhoI* restriction sites and in the adjacent position to a C-terminal His₆-tag-encoding region. Proteins were overexpressed in the *Escherichia coli* Origami 2 (DE3) strain (Novagen). Protein sequences for the construct were chosen on a homology basis with other proteins containing the PA14 domain (Veelders *et al.*, 2010). Cells were grown at 310 K until an OD_{600 nm} of

0.8 was reached. The temperature was then decreased to 285 K and protein expression was induced by adding isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 50 μM . After 2 d of incubation, the cells were harvested by centrifugation for 30 min at 4000g and 277 K, and were resuspended in buffer 1 (20 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ pH 7.4, 1 M NaCl, 45 mM imidazole). Cells were lysed with a cell homogenizer and the lysate was centrifuged for 30 min at 20 000g and 277 K. The supernatant was applied onto an Ni-affinity column (His-Trap HP 1 ml, GE Healthcare) pre-equilibrated with buffer 1. An isocratic elution of the protein was performed with buffer 2 (50 mM Tris–HCl pH 7.5, 200 mM NaCl, 500 mM imidazole). Fractions containing N-Flo1p or N-LgFlo1p were pooled and dialysed against buffer 3 (50 mM Tris–HCl pH 7.5), in order to reach a conductivity lower than 5 mS. Proteins were then subjected to a second purification step by applying them onto a column packed with Source 15Q anion-exchange beads (GE Healthcare), which was pre-equilibrated first with buffer 4 (50 mM Tris–HCl pH 7.5, 1 M NaCl) and then with a double volume of buffer 3. The protein was eluted with a gradient of buffer 4 from 0 to 100% in 40 column volumes. Fractions were pooled, dialysed against 50 mM Tris–HCl, 200 mM NaCl pH 7.5 and concentrated. Protein concentration (FW N-Flo1p, 28690 Da; FW N-LgFlo1p, 26640 Da) was determined spectroscopically by measuring the sample absorbance at 280 nm.

2.2. Crystallization

2.2.1. N-Flo1p crystallization. The crystal screens Morpheus, JCSG+ HT96 (Molecular Dimensions) and Crystal Screen HT (Hampton Research) were chosen for an initial screening of crystallization conditions. Screen plates were prepared using 96-well Intelli-Plates for the sitting-drop method using a Phoenix Liquid Handling System robot (Art Robbins Instruments). After exchange of the N-Flo1p buffer by dialysis against 50 mM acetate buffer pH 5, 150 mM NaCl, 10 mM CaCl_2 , crystallization was tested at 293 K for a protein solution with a concentration of 10 mg ml^{−1}. Small polyhedral crystals were found after 1 week in multiple hit conditions from all screens. Crystals obtained from hit conditions were collected from the respective wells with a cryoloop, soaked in a drop of mother liquor containing 20% (v/v) glycerol as a cryoprotectant and stored in liquid nitrogen for subsequent X-ray data collection. One of the hit conditions from Crystal Screen HT [1.5 M NaCl, 10% (v/v) EtOH] was further optimized by varying the ethanol (from 10 to 40%) and NaCl concentrations (from 0 to 2 M). For the optimization screens, 48-well plates (Becton Dickinson) and the hanging-drop method were used. Drops were prepared by mixing 1 μl protein solution (10 mg ml^{−1}) with 1 μl screen solution taken from the 350 μl in the reservoir. Crystallization was successfully achieved at a temperature of 293 K in about 1 week in conditions containing 10 or 15% (v/v) ethanol and between 0.5 and 2 M NaCl (Fig. 1a). For the apo N-Flo1p structure determination, crystals from optimization screening were soaked in a drop of mother liquor containing 20% (v/v) glycerol as a cryoprotectant and were stored in liquid nitrogen for subsequent X-ray data collection. For the N-Flo1p–mannose structure determination, crystals were soaked in a drop of mother liquor containing 130 mM D-mannose, collected with a MicroMount and inserted into a MicroRT plastic capillary pre-filled with the mother liquor as a humidity-stabilizing liquid. The MicroMount and the MicroRT capillary are part of the MicroRT Room Temperature starter kit (MiTeGen) for the collection of X-ray diffraction data at room temperature.

Figure 1
Crystals of the flocculin N-terminal domains. (a) Crystals of apo N-Flo1p from one optimization screening hit: 2 M NaCl, 15% (v/v) EtOH. (b) Crystals of apo N-Lg-Flo1p grown in 0.1 M amino acids (sodium L-glutamate, DL-alanine, glycine, DL-lysine-HCl, DL-serine), 0.1 M imidazole-MES pH 6.5, 30% (w/v) PEG 550 MME-PEG 20000.

2.2.2. N-Lg-Flo1p crystallization. The Morpheus crystal screen (Molecular Dimensions) was chosen for an initial screening of crystallization conditions. Screen plates were manually prepared using 48-well plates for the hanging-drop method. Crystallization was

tested at 293 K for an N-LgFlo1p solution with a concentration of 18 mg ml^{-1} in 50 mM Tris-HCl pH 7.5, 200 mM NaCl and containing an additional 10 mM CaCl_2 . Bundles of rod-shaped crystals were obtained in one screen condition [0.1 M amino acids (sodium L-glutamate, DL-alanine, glycine, DL-lysine-HCl and DL-serine), 0.1 M imidazole-MES pH 6.5, 30% (w/v) PEG 550 MME-PEG 20000] about 1 month after screen preparation (Fig. 1b). A few crystals were detached from the initial bundle, soaked for a few minutes in a drop of mother liquor containing 20% (v/v) glycerol and 0.5 M α -1,2-mannobiose and then stored in liquid nitrogen for subsequent X-ray data collection.

2.3. Data collection and processing

Diffraction data were collected at 100 K with a PILATUS 6M detector on beamline PROXIMA1 at the SOLEIL synchrotron (N-Lg-Flo1p-mannobiose complex) or with an ADSC Quantum 315r detector on beamline I04 at the Diamond synchrotron (apo-N-Flo1p) (Figs. 2a, 2c). For the N-Flo1p-mannose complex, data were collected at room temperature (298 K) with an in-house Rigaku MicroMax-007 HF X-ray generator equipped with a Saturn 944+ detector (Fig. 2b). Data were processed with *XDS* (Kabsch, 2010). Space-group confirmation and scaling were performed with *POINTLESS* and *AIMLESS*, respectively, which are included in the *CCP4* program suite (Winn *et al.*, 2011). Data from the apo-N-Flo1p crystal were processed with the *xia2* automated system (Winter, 2010) using *XDS*. The data-collection statistics are indicated in Table 1.

3. Results and discussion

N-Flo1p shares high sequence identity (93%) with the previously characterized N-Flo5p (Veelders *et al.*, 2010; PDB entry 2xjq). Nonetheless, determination of its three-dimensional structure, either ligand-free or in complex with carbohydrates, is necessary for a thorough study of the flocculation process. The apo form of the N-Lg-Flo1p structure has been recently determined (Sim *et al.*, 2013; PDB entry 4gq7), but no X-ray diffraction data for any Lg-Flo1p-carbohydrate complex are available in the literature.

Our objective is to characterize in detail and at a molecular level the flocculation phenomenon in *Saccharomyces* species. Investigation

Figure 2
X-ray diffraction patterns. High-resolution limits are marked on the figure and the respective values are reported in Å in the bottom right corner of each panel. (a) Apo N-Flo1p. (b) N-Flo1p-mannose. (c) N-Lg-Flo1p- α -1,2-mannobiose.

Table 1

Data-collection statistics for the N-Flo1p and N-Lg-Flo1p crystals.

Values in parentheses are for the outermost resolution shell.

Crystal	Apo N-Flo1p	N-Flo1p-mannose	N-Lg-Flo1p- α -1,2-mannobiose
Wavelength (Å)	0.97950	1.54	0.98011
Space group	$P2_12_12_1$	$P2_12_12_1$	$P12_11$
Unit-cell parameters (Å, °)	$a = 45.800$, $b = 61.770$, $c = 106.730$, $\alpha = \beta = \gamma = 90$	$a = 46.70$, $b = 63.59$, $c = 106.54$, $\alpha = \beta = \gamma = 90$	$a = 38.62$, $b = 85.48$, $c = 68.24$, $\alpha = \gamma = 90$, $\beta = 93.72$
Matthews coefficient (Å ³ Da ⁻¹)	2.63	2.76	2.11
Solvent content (%)	53.3	55.4	41.7
Resolution (Å)	61.77–1.43 (1.47–1.43)	15.37–2.12 (2.23–2.12)	38.54–1.73 (1.76–1.73)
No. of unique reflections	56735 (4114)	16718 (2106)	44852 (2430)
Completeness (%)	99.9 (99.8)	90.1 (80.3)	97.3 (96)
Multiplicity	7.2 (7.2)	5.7 (5.6)	4.5 (4.7)
$\langle I/\sigma(I) \rangle$	13.6 (3.0)	12.3 (3.7)	9.9 (2.1)
$R_{\text{merge}}^{\dagger}$ (%)	8.5 (64.7)	10 (43.3)	9.2 (70.4)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity of multiple observations of symmetry-related reflections.

of the structural features of the N-terminal lectin domains of adhesins in their bound state is fundamental in order to define the mechanism underlying this cellular self-aggregation process.

N-Flo1p and N-Lg-Flo1p were both produced and purified from the *E. coli* Origami 2 strain, which is ideal for slow low-temperature expression of proteins containing disulfide bonds. Additionally, this *E. coli* expression system is more suitable for crystallization than the *Pichia pastoris* system which was previously used for N-Lg-Flo1p (Groes *et al.*, 2002). A functional, homogeneous and nonglycosylated protein is produced in this case, which does not require any treatment with endoglycosidases prior to preparation for crystallization screening.

N-Flo1p crystals were easily obtained in several screen conditions. The morphology and quality of the crystals from the initial conditions were suitable for X-ray analysis. However, optimization of the screen conditions was attempted in order to improve the dimensions and quality of the apo-N-Flo1p crystals. Most of the initial screen hits included EtOH and NaCl in their composition, either separately or mixed, at different pH values. Thus, an optimization screening including ethanol [10–40% (v/v)] and salt (0–2 M) was set up. A large number of polyhedral crystals, similar to those obtained in the initial conditions, formed in the presence of at least 0.5 M NaCl and with 10 or 15% (v/v) EtOH (Fig. 1b).

All N-Flo1p crystals belonged to the orthorhombic space group $P2_12_12_1$ with one molecule in the asymmetric unit. Different crystals from the initial and the optimization screenings were collected and analysed. The best data quality was obtained for apo N-Flo1p crystals from one optimization screen condition [2 M NaCl, 10% (v/v) EtOH]. In this case, a data set was collected to 1.43 Å resolution and the unit-cell parameters were $a = 45.800$, $b = 61.770$, $c = 106.730$ Å. The high specificity of the Flo1p lectin domain for mannose and mannosides has previously been determined (Van Mulders *et al.*, 2010; Goossens *et al.*, 2011). Soaking of N-Flo5p crystals in different monosaccharide or oligosaccharide ligand solutions has been reported as a simple, convenient and successful strategy for the structural study of flocculin-carbohydrate complexes (Veelders *et al.*, 2010). In our case, room-temperature diffraction from a crystal grown in one optimization condition [1 M NaCl, 15% (v/v) EtOH] and soaked in a D-mannose solution resulted in a data set to 2.12 Å resolution and

unit-cell parameters of $a = 46.70$, $b = 63.59$, $c = 106.54$ Å. The same space group and similar unit-cell parameters were found for one of the N-Flo5p crystal forms (Veelders *et al.*, 2010).

Crystallization of deglycosylated N-Lg-Flo1p was achieved more than a decade ago. Groes *et al.* (2002) reported preliminary X-ray diffraction data from needle-shaped crystals obtained from micro-seeding optimization experiments. Likewise, our N-Lg-Flo1p crystallization screening yielded multinucleated bundles of elongated crystals (Fig. 1c), but they were characterized by a different space group. We obtained a single hit condition that gave monoclinic $P12_11$ crystals with unit-cell parameters $a = 38.62$, $b = 85.48$, $c = 68.24$ Å, $\alpha = \gamma = 90$, $\beta = 93.72^\circ$. A complete diffraction set was collected to a resolution of 1.73 Å. Before the collection of diffraction data, the crystal was soaked in a solution of α -1,2-mannobiose, which is one of the flocculin ligands.

We successfully purified and crystallized the adhesins N-Flo1p and N-Lg-Flo1p from *S. cerevisiae* and *S. pastorianus*. Structure determination by molecular replacement, using the known homologous structure of N-Flo5p, and refinement of the ligand-free and carbohydrate-bound solutions are in progress.

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