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Crystal structure of recombinant tyrosinase-binding protein MtaL at 1.35 Å resolution

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Mushroom tyrosinase-associated lectin-like protein (MtaL) binds to mature *Agaricus bisporus* tyrosinase *in vivo*, but the exact physiological function of MtaL is unknown. In this study, the crystal structure of recombinant MtaL is reported at 1.35 Å resolution. Comparison of its structure with that of the truncated and cleaved MtaL present in the complex with tyrosinase directly isolated from mushroom shows that the general β -trefoil fold is conserved. However, differences are detected in the loop regions, particularly in the $\beta 2$ – $\beta 3$ loop, which is intact and not cleaved in the recombinant MtaL. Furthermore, the N-terminal tail is rotated inwards, covering the tyrosinase-binding interface. Thus, MtaL must undergo conformational changes in order to bind mature mushroom tyrosinase. Very interestingly, the β -trefoil fold has been identified to be essential for carbohydrate interaction in other lectin-like proteins. Comparison of the structures of MtaL and a ricin-B-like lectin with a bound disaccharide shows that MtaL may have a similar carbohydrate-binding site that might be involved in glycoreceptor activity.

1. Introduction

Tyrosinase (EC 1.14.18.1) is a binuclear copper-containing enzyme that catalyzes the *o*-hydroxylation of monophenols to the corresponding *o*-diphenols and the subsequent conversion of the *o*-diphenols to the corresponding *o*-quinones (Burton, 2003; Claus & Decker, 2006). *o*-Quinones are precursors for the synthesis of melanins, which are pigments that play important roles in the survival of organisms ranging from bacteria and plants to mammals (Kitajima & Moro-oka, 1994; Solomon *et al.*, 1996; Marusek *et al.*, 2006). In mammals, melanin is mostly found in the skin, where it functions in photoprotection against UV radiation. Plants employ *o*-quinones in modifying and hardening the protective exterior layer of, for instance, seed envelopes, and as agents against invasive organisms. The latter function also occurs in fruits and potatoes and in the fruit bodies of fungi. These examples illustrate that the function of tyrosinase is associated with a response to adverse stress influences from the environment (Land *et al.*, 2004).

Tyrosinase can be isolated from the common button mushroom *Agaricus bisporus* as a heterotetrameric protein with a total molecular mass of 120 kDa consisting of two H chains (~43 kDa each) and two L chains (~14 kDa each) (Strothkamp *et al.*, 1976; Ismaya *et al.*, 2011). The enzyme is produced as an inactive precursor with a molecular mass of

66 kDa, which led to the suggestion that maturation occurs through the proteolytic removal of a ~ 20 kDa fragment by either endogenous or pathogen (serine) proteases (Espín & Wichers, 1999). Since the L chain has a molecular mass of 17 kDa, it was initially assumed to be part of the full-length tyrosinase. However, the crystal structure of the H_2L_2 complex (PDB entry 2y9w; Ismaya *et al.*, 2011) revealed that the L chain is not derived from the tyrosinase precursor, but is the product of a completely different *A. bisporus* gene, ORF239342, and has a lectin-like fold. Lectin proteins exert their activity by binding to specific glycoreceptors (Pohleven *et al.*, 2012). Remarkably, the L chain is unique to *A. bisporus* and clusters close to the tyrosinase genes, which suggest that this cofactor is not an artifact from biochemical tyrosinase purification but has a real biological function in the pathways involving tyrosinase in *A. bisporus* (Weijn *et al.*, 2013). Unfortunately, only a truncated and cleaved form of the ORF239342 product was present in the crystals of mushroom tyrosinase, which precluded firm conclusions on the role of the

L subunit as well as its importance for the functioning of the H subunit.

Here, we present the structure of the intact, full-length mushroom tyrosinase-associated lectin-like protein (hereafter called MtaL) at 1.35 Å resolution and show that conformational changes are needed to bind the tyrosinase H subunit. Furthermore, a structural alignment with proteins containing a lectin-like fold reveals a putative carbohydrate-binding site that may be associated with glycoreceptor activity.

2. Materials and methods

2.1. Cloning, expression and purification

The gene encoding the L-chain protein was codon-optimized for *Escherichia coli* and synthesized by Shine Gene Molecular Biotech Inc., Shanghai, People's Republic of China. The gene was inserted into the pUC57 vector and amplified by the polymerase chain reaction, using L_NheI_for (5'-AA-TTGCTAGCATGGCTCAGGCTCGTAAAATC-3') and L_NotI_Tev_rev (5'-AATTGCGGCCGCTCCCTGAAAATACAAATTCTCAACAGCGAATTTGATACGC-3') as primers, which introduced NheI and NotI restriction sites (underlined), respectively, and a carboxyl-terminal *Tobacco etch virus* cleavage site. The generated PCR product was digested with the NheI and NotI restriction endonucleases and the product was purified and ligated into the pET-21d(+) vector digested with the same restriction enzymes. The resulting construct containing the L-chain gene was confirmed by sequencing (Macrogen Inc., Amsterdam, The Netherlands).

For expression, the pET construct was used to transform *E. coli* BL21 (DE3) cells (Invitrogen) containing an isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible T7 polymerase gene. Transformants were grown overnight at 310 K in Luria-Bertani medium to an optical density at 600 nm (OD_{600}) of 0.8–1.0 in the presence of 50 $\mu\text{g ml}^{-1}$ ampicillin. Expression of the recombinant protein was induced by 0.2 mM IPTG at 303 K. The cells were harvested by centrifugation at 6000g for 15 min, resuspended in binding buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl) and disrupted by sonication on ice by applying 10 s bursts with 10 s cooling intervals for 15 min. After centrifugation at 20 000g for 30 min, the supernatant was collected and filtered through a 0.45 μm pore-size membrane filter. Subsequently, the supernatant was applied onto Ni²⁺-nitrilotriacetate affinity resin (Ni-NTA, Qiagen) and incubated for 20 min in a cold room. The resin was washed with binding buffer supplemented with 50 mM imidazole. The bound protein was eluted with elution buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 500 mM imidazole) and the His₆ tag was removed by overnight incubation at 273 K with TEV protease at a TEV protease:MtaL molar ratio of 1:50. The cleaved sample was concentrated and applied onto a Superdex 200 column (GE Healthcare, Buckinghamshire, England) pre-equilibrated with 25 mM Tris buffer pH 8.0, 50 mM NaCl for a final size-exclusion chromatography purification step. The protein eluted in a single peak corresponding to a molecular weight of ~ 17 kDa (Fig. 1a). The homogeneity of the protein

Figure 1
(a) Superdex 200 gel-filtration column chromatogram showing a peak at an apparent molecular mass of ~ 17 kDa. (b) SDS-PAGE gel of peak fractions of the gel-filtration chromatography purification step. The left lane contains molecular-mass markers (labelled in kDa).

Table 1

Data-collection and processing statistics.

Values in parentheses are for the outer shell.

Diffraction source	ID29, ESRF
Wavelength (Å)	0.976
Temperature (K)	100
Space group	<i>I</i> 121
<i>a</i> , <i>b</i> , <i>c</i> (Å)	56.97, 61.34, 59.05
α , β , γ (°)	90, 108.36, 90
Mosaicity (°)	0.06
Resolution range (Å)	47.01–1.35 (1.42–1.35)
Total No. of observed reflections	168208 (24357)
No. of unique reflections	41973 (6084)
Completeness (%)	99.2 (98.8)
$\langle I/\sigma(I) \rangle$	16.9 (2.7)
$R_{\text{merge}}^{\dagger}$	0.038 (0.455)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an observation, $\langle I(hkl) \rangle$ is the mean value for that reflection and the summations are over all equivalents.

was confirmed by SDS–PAGE (Fig. 1*b*). Fractions containing pure protein were pooled and concentrated to $\sim 20 \text{ mg ml}^{-1}$ for crystallization screening. The purified proteins were stored at 193 K.

2.2. Crystallization

Screening for suitable crystallization conditions was performed with an HTX crystallization robot (EMBL Grenoble, France) using both commercial and home-made crystallization screening solutions. The crystallization screens were set up by mixing 100 nl protein solution and 100 nl reservoir solution in 96-well sitting-drop plates. All crystallization trials were performed at 293 K. The most promising initial crystallization results were obtained in a condition consisting of 0.1 *M* bis-tris buffer pH 5.5, 2 *M* ammonium

Table 2

Structure solution and refinement.

Resolution range (Å)	47.01–1.35
<i>R</i> factor	0.162
Free <i>R</i> factor	0.183
R.m.s.d., bond lengths (Å)	0.027
R.m.s.d., bond angles (°)	2.432
No. of protein atoms	1230
No. of water molecules	219
Average <i>B</i> factor (Å ²)	
Protein	21.7
Water	35.6
Ramachandran analysis	
No. of residues in favoured regions	153
No. of residues in disallowed regions	0

sulfate as a precipitant in the reservoir (Index screen condition No. 3, Hampton Research; Fig. 2*a*). This condition was manually optimized by mixing 1 μl protein solution with 1 μl reservoir solution and varying the pH and the ammonium sulfate concentration. Rod-like crystals were obtained from a setup with reservoir solution consisting of 0.1 *M* bis-tris buffer pH 5.6, 2.1 *M* ammonium sulfate (Fig. 2*a*, bottom).

2.3. Data collection, processing, structure solution and refinement

The crystals were mounted in a cryo-loop and subsequently flash-cooled in liquid nitrogen. X-ray diffraction data were collected at 100 K on beamline ID29 (wavelength 0.976 Å) at the European Synchrotron Radiation Facility (ESRF), Grenoble, France using a Pilatus 6M-F detector (Dectris) at a distance of 235.10 mm. The monoclinic crystal was rotated through 213° with an oscillation range of 0.05° per frame. The crystal diffracted to a resolution of 1.35 Å (Fig. 2*b*). The data

Figure 2

(*a*) Crystallization hit from the HTX crystallization robot (EMBL Grenoble, France) using the sitting-drop vapour-diffusion method. The bottom figure shows the rod-like crystals obtained after optimization. (*b*) Diffraction pattern of an MtaL crystal. The resolution at the edge of the detector is 1.32 Å and the arrow at the bottom left indicates spots at 1.35 Å resolution.

set was processed and integrated using *XDS* (Kabsch, 2010) in combination with *SCALA* (Evans, 2006) from the *CCP4* package (Winn *et al.*, 2011) (Tables 1 and 2). The structure was phased using molecular replacement with *Phaser* (McCoy *et al.*, 2007) and the structure of the L chain from the mushroom tyrosinase complex (PDB entry 2y9w, chain C; Ismaya *et al.*, 2011) as the search model. Manual rebuilding was performed with *Coot* (Emsley *et al.*, 2010) and refinement was performed with *REFMAC5* (Murshudov *et al.*, 2011). The coordinates and structure factors have been deposited in the PDB with accession code 5eha.

3. Results and discussion

3.1. Overall structure of the MtaL protein

MtaL has a β -trefoil fold consisting of 12 antiparallel β -strands assembled in a cylindrical barrel of six two-stranded sheets (Fig. 3). The full-length structure covers residues Ala4–Val150; no electron density is visible for the first three residues (MAQ) or the extra residues (ENLYFQ) introduced by the TEV cleavage site at the C-terminus. Residues 29–34 (loop $\beta 2$ – $\beta 3$; Fig. 3), which were not visible in the L chain in the H_2L_2 complex structure, likely because of proteolytic cleavage (Ismaya *et al.*, 2011), are well defined in the electron density and are not cleaved (Fig. 4a). Their conformation is stabilized through crystal-packing interactions with residues from a symmetry-related molecule (indicated by an asterisk), with hydrogen bonds between Asn30 N ^{$\delta 2$} and Glu103 O ^{$\epsilon 1$ *}, Asn30 O ^{$\delta 1$} and Glu103 N*, Leu31 N and Glu104 O ^{$\epsilon 1$ *}, and Ala32 N and Glu104 O ^{$\epsilon 2$ *} (Fig. 4b). Nevertheless, the $\beta 2$ – $\beta 3$ loop is probably intrinsically flexible since in a different crystal structure of MtaL in space group $P2_1$ it shows poor density for

some residues that are not stabilized by a symmetry-related molecule (data not shown). Whether this loop conformation accounts for a specific interface with the $\beta 8$ – $\beta 9$ loop from an adjacent MtaL molecule or simply results from an artifact of crystal packing remains unclear.

3.2. Conformational changes upon binding to mushroom tyrosinase

The structure of full-length monomeric MtaL is similar to that of the L-chain subunit bound to mushroom tyrosinase in the H_2L_2 complex that was solved previously (PDB entry 2y9w, chain C; Ismaya *et al.*, 2011), with a Z-score of 25.8 and an r.m.s.d. of 0.9 Å for 136 superimposed C α atoms. No major conformational differences were observed between the free and tyrosinase-bound L subunits (Fig. 5a). However, a flexible loop consisting of residues Ala4–Gly14 is rotated by 90° into the core of the protein, preventing solvent exposure of the residues required for specific binding to tyrosinase (Fig. 5b).

3.3. Putative carbohydrate-binding site

A search for structural homologues using *DALI* (Holm *et al.*, 2008) revealed that MtaL has the closest structural similarity to the neurotoxin-associated haemagglutinating protein HA33 from *Clostridium botulinum*, with a Z-score of 16.9, an r.m.s.d. of 1.9 Å for 129 aligned residues and 14% sequence identity (PDB entry 1ybi, chain A; Arndt *et al.*, 2005). MtaL has the highest sequence identity to the ricin-B-like lectin CNL from the basidiomycete *Clitocybe nebularis*, with 22% sequence identity, a Z-score of 16.5 and an r.m.s.d. of 2.6 Å for 130 aligned residues (PDB entry 3nbe, chain A; Pohleven *et al.*, 2012; Fig. 6a). The two homologues are both carbohydrate-binding proteins. MtaL also shares structural homology with

Figure 3

Cartoon diagram of the *A. bisporus* MtaL crystal structure rainbow colour-coded from the N-terminus (blue) to the C-terminus (red) showing the 12 β -strands adopting a β -trefoil domain organization. The diagram on the right shows the crystal structure rotated by 90° around the vertical axis.

the sea mussel galectin CGL from *Crenomytilus grayanus* (PDB entry 5duy, chain A; 8% sequence identity, Z-score of 13.5, r.m.s.d. of 2.4 Å for 122 aligned residues), which is specific for binding GalNAc/Gal-containing carbohydrate moieties, although it does not share sequence homology with

other known galectins or lectins (Jakób *et al.*, 2015). Interestingly, a structural alignment of MtaL with CNL with bound α -lactose (PDB entry 3nbd, chain B; Pohleven *et al.*, 2012) shows similar residues in the sugar-binding site (Fig. 6b). Only two hydroxyl groups, both belonging to the galactosyl moiety

Figure 4
(a) Electron density of residues 29–34 from the β 2– β 3 loop and (b) their interactions with a symmetry-related MtaL molecule (highlighted in grey), showing the stabilizing hydrogen bonds. The $2F_o - F_c$ map is contoured at 1σ and shown as a blue mesh.

Figure 5
(a) Superposition of MtaL (orange) with the L chain of the H_2L_2 complex (green). The cleavage site in the β 2– β 3 loop and the N- and C-termini are indicated. (b) Close-up of the tyrosinase-binding interface, showing the N-terminal tail of MtaL (red) rotated by about 90° inwards compared with the equivalent N-terminal tail (green) in the H_2L_2 tyrosinase complex (purple).

of α -lactose, make hydrogen bonds to the CNL protein: the O3 and O4 hydroxyl groups interact with the Asp20 carboxylate, with additional interactions with the N⁸² atom of Asn38 (O3), the N⁸² atom of Asn46 (O3) and the main-chain N atom of Gly23 (O4) (Fig. 6*b*, bottom right). No stacking interactions

with aromatic side chains are present. Notably, the CNL structure in complex with a nonreducing *N*-acetylgalactosamine (GalNAc)-containing carbohydrate (LDN; PDB entry 3nbe) shows a similar binding to that of lactose, although one additional hydrogen bond is formed between the O2-bound

Figure 6

(a) Structure-based sequence alignment of MtaL with the ricin-B-like lectin (CNL; PDB entry 3nbd, chain B), haemagglutinating protein (HA33; PDB entry 1ybi, chain B) and the sea mussel lectin (CGL; PDB entry 5duy, chain A) performed with the *SALIGN* web server (Braberg *et al.*, 2012). Carbohydrate-binding residues are highlighted in red. (b) Surface representation of a superposition of the MtaL (gold) and ricin-B-like lectin (cyan) crystal structures. The binding of lactose in the CNL carbohydrate-binding pocket is shown as a stick model. A close-up view of the putative MtaL sugar-recognition/binding site based on the CNL structural alignment is shown at the bottom left of the figure. Loops $\beta 2$ – $\beta 3$ and $\beta 3$ – $\beta 4$ involved in CNL–sugar binding could also play a similar role in MtaL, where Pro46 in the $\beta 3$ – $\beta 4$ loop (Pro41 in CNL) must rotate to enable sugar accommodation. The bottom right figure shows the potential sugar-binding residues that could provide hydrogen-bonding interactions: Asn24 (equivalent to Asp20 in CNL), Asp42 (equivalent to His35) and Thr45 (equivalent to Asn38), highlighted in pink and dark blue, respectively.

carbonyl O atom in the acetate group of GalNAc and the hydroxyl O atom of Ser24 (Pohleven *et al.*, 2012). At the equivalent position in MtaL, sufficient space is also available for binding a saccharide. Among the residues that could provide hydrogen-bonding interactions are Asn24 (equivalent to Asp20 in CNL), Asp42 (equivalent to His35) and Ser26 (equivalent to Ser24). Furthermore, loops $\beta 2$ – $\beta 3$ and $\beta 3$ – $\beta 4$, which are involved in sugar binding in CNL, could have a similar role in MtaL. In particular, the $\beta 3$ – $\beta 4$ loop contains a proline residue (Pro46) that must move out of the way to accommodate a sugar molecule, similar to what was observed for its counterpart Pro41 in the lactose-bound CNL structure (Fig. 6*b*, bottom left). Movement of the $\beta 3$ – $\beta 4$ loop would enable Thr45 (equivalent to Asn38 in CNL) to make hydrogen-bonding interactions with the sugar. Nevertheless, extensive crystal-soaking studies to obtain a structure of MtaL with a bound carbohydrate (such as lactose, glucose, raffinose, galactose or sucrose) were unsuccessful. Thus, although it is an attractive hypothesis that MtaL binds carbohydrates like other lectin-like-fold proteins (Arndt *et al.*, 2005), so far no experimental evidence for such a role has been obtained.

4. Conclusions

In conclusion, our findings indicate that MtaL undergoes conformational changes to bind tyrosinase. Whether the proteolytic cleavage of the protruding $\beta 2$ – $\beta 3$ loop has biological relevance requires further investigation (Espín & Wichers, 1999; Ismaya *et al.*, 2011). Furthermore, MtaL might have a potential carbohydrate-binding site based on structural analyses, although its functional activity remains unclear. Taken together, these results provide insights into the structural mechanisms of tyrosinase recognition by MtaL and shed light on its biological function.

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