Structure and activity studies of tyrosinases and related proteins

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Chapter 2. Crystal structure of recombinant tyrosinase-binding protein MtaL at 1.35 Å resolution

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Abstract

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, we report the crystal structure of recombinant MtaL 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 β2-β3 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 and Decker, 2006). O-quinones are precursors for the synthesis of melanos, which are pigments that play important roles in the survival of organisms ranging from bacteria, plants, to mammals (Kitajima and Moro-oka, 1994; Marusek et al., 2006; Solomon et al., 1996). 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 potato, 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) (Ismaya et al., 2011; Strothkamp et al., 1976). The enzyme is produced as an inactive precursor with a molecular mass of 66 kDa per peptide chain, 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 and 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 H2L2 complex (PDB code 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 \textit{A. bisporus} gene, \textit{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 for \textit{A. bisporus} and clusters close to the tyrosinase genes, which suggest that this co-factor is not an artifact from biochemical tyrosinase purification but has a real biological function in the tyrosinase-involved pathways in \textit{A. bisporus} (Weijn et al., 2013). Unfortunately, only a truncated and cleaved form of the \textit{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 to 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.

\section*{2. Materials and methods}

\subsection*{2.1 Cloning, expression and purification}

The gene encoding the L chain protein was codon optimized for \textit{E. coli} and synthesized by Shine Gene Molecular Biotech Inc. (Shanghai, China). The gene was inserted into the pUC57 vector, and amplified by the polymerase chain reaction, using as primers \texttt{L}\_\texttt{Nhe1}\_\texttt{for} (5'\- AATTGCTAGCATGGCTAGGTCAAAAAATATG-3') and \texttt{L}\_\texttt{Not1}\_\texttt{Tev}\_\texttt{rev} (5'\- AATTGCGGCCGCTCCCTGAAAAATACAAATTCTCAACAGGAATTTGATACGC-3'), which introduced \textit{Nhe1} and \textit{Not1} restriction sites (underlined), respectively, and a carboxyl-terminal Tobacco Etch Virus cleavage site. The generated PCR product was digested with the \textit{Nhe1} and \textit{Not1} restriction endonucleases and the product was purified and ligated into the pET21d+ vector digested with the same restriction enzymes. The resulting construct containing the L chain gene was confirmed by sequencing (Macrogen Inc., Amsterdam).

For expression, the pET construct was used to transform \textit{E. coli} BL21 (DE3) cells (Invitrogen) containing an isopropyl-\(\beta\)-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 (OD600) of 0.8-1.0 in the presence of 50 \(\mu\)g ml\(^{-1}\) ampicillin. The expression of the recombinant protein was induced by 0.2 mM IPTG at 303 K. Cells were harvested by centrifugation at 6000g for 15 min and resuspended in binding buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl), and disrupted by sonication on ice by applying 10-second bursts with 10-second cooling intervals for 15 min. After centrifugation at 20000g for 30 min, the supernatant was collected and filtered through a 0.45 \(\mu\)m pore size membrane filter. Subsequently, the supernatant was applied to a Ni\(^{2+}\)-
nitrilotriacetate affinity resin (Ni-NTA, Qiagen) and incubated for 20 min in the 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 to 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 of ~17 kDa molecular weight (Figure 1a). The homogeneity of the protein was confirmed by SDS-PAGE (Figure 1b). Fractions with pure protein were pooled and concentrated to ~20 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 the HTX crystallization robot (EMBL-Grenoble, France) using both commercial and homemade 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 done at 293 K. The most promising initial crystallization results were obtained in a condition containing 0.1 M bis-tris buffer, pH 5.5, and 2 M ammonium sulfate as a precipitant in the reservoir (Index screen, condition #3, Hampton Research) (Figure 2a). This condition was manually optimized by mixing 1 µl protein solution with 1 µl reservoir solution, and varying the pH and ammonium sulfate concentration. Rod-like crystals were obtained from a setup with reservoir solution containing...
0.1 M bis-tris buffer, pH 5.6, and an ammonium sulfate concentration of 2.1 M (Figure 2a, bottom figure).

2.3 Data collection, processing, structure solution and refinement

The crystals were mounted in a cryo loop and subsequently flash-frozen in liquid nitrogen. X-ray diffraction data were collected at 100 K on beam line ID29 (wavelength 0.976 Å) at the European Synchrotron Radiation Facility (Grenoble, France) using a Pilatus 6M-F detector (DECTRIS) at a distance of 235.10 mm. The monoclinic crystal was rotated over 213° with an oscillation range of 0.05° per frame. The crystal diffracted to a resolution of 1.35 Å (Figure 2b). The data set was processed and integrated using the program XDS (Kabsch, 2010) in combination with the program SCALA (Evans, 2006) from the CCP4 package (Winn et al., 2011) (Tables 1 and 2). The structure was phased using molecular replacement with the program Phaser (McCoy et al., 2007) and the structure of the L chain from the mushroom tyrosinase complex (PDB code 2Y9W, chain C (Ismaya et al., 2011)) as the search model. Manual rebuilding was performed with COOT (Emsley et al., 2010) and refinement was done with REFMAC5 (Murshudov et al., 2011). The coordinates and structure factors have been deposited in the PDB with accession code 5EHA.

Figure 2. (a) Crystallization hit from the HTX crystallization robot (EMBL-Grenoble, France) using the sitting-drop vapor 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.
Table 1 Data collection and processing statistics (Values for the outer shell are given in parentheses).

<table>
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<tr>
<td>Temperature</td>
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<td>Space group</td>
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<td>$R_{merge}$ †</td>
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$R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, 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.

Table 2 Structure solution and refinement.

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<tr>
<td>Number of residues in disallowed regions</td>
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3. Results and discussion

3.1 Overall structure of the MtaL protein
MtaL has a β-trefoil fold, consisting of 12 anti-parallel β-strands assembled in a cylindrical barrel of six 2-stranded sheets (Figure 3). The full-length structure
covers residues Ala4 to Val150; no electron density is visible for the first three residues (MAQ) and the extra residues (ENLYFQ) introduced by the TEV cleavage site at the C-terminus. Residues 29-34 (loop β2-β3, Figure 3), which were not visible in the L chain in the H2L2 complex structure, likely because of proteolytic cleavage (Ismaya et al., 2011), are well defined in the electron density and are not cleaved (Figure 4a). Their conformation is stabilized through crystal packing interactions with residues from a symmetry-related molecule (indicated by *), with hydrogen bonds between Asn30 Nδ2 and Glu103 Oε1*, Asn30 Oδ1 and Glu103 N*, Leu31 N and Glu104 Oε1*, Ala32 N and Glu104 Oε2* (Figure 4b). Nevertheless, the β2-β3 loop is probably intrinsically flexible since in a different crystal structure of MtaL in space group P21 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 β8-β9 loop from an adjacent MtaL molecule or simply results from an artifact of crystal packing remains unclear.

Figure 3. Cartoon diagram of the Agaricus bisporus MtaL crystal structure rainbow color-coded from N-terminus (blue) to 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.
Figure 4. (a) Electron density of residues 29-34 from the β2-β3 loop, and (b) their interactions with a symmetry-related MtaL molecule (highlighted in grey), showing the stabilizing hydrogen bonds. The 2Fo-Fc map is contoured at 1σ and shown as a blue mesh.

Figure 5. (a) Superposition of MtaL (orange) with the L chain of the H2L2 complex (green). The cleavage site in the β2-β3 loop and the N- and C-terminus are indicated. (b) Close-up of the tyrosinase-binding interface, showing the N-terminal tail of MtaL (red) rotated by about 90° inwards compared to the equivalent N-terminal tail (green) in the H2L2 tyrosinase complex (purple).

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 H2L2 complex that was solved before (PDB code 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 Cα atoms. No major conformational differences were observed between the free and tyrosinase-bound L subunits (Figure 5a). However, a flexible loop consisting of residues
Ala4 to Gly14 is rotated by 90° into the core of the protein, preventing solvent exposure of the residues required for specific binding to tyrosinase (Figure 5b).

3.3 Putative carbohydrate-binding site

A structural homologue search using DALI (Holm et al., 2008) revealed that MtaL has the closest structural similarity to the neurotoxin-associated hemagglutinating 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 code 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 and a Z-score of 16.5, and an r.m.s.d. of 2.6 Å for 130 aligned residues (PDB code 3NBE, chain A (Pohleven et al., 2012)) (Figure 6a). The two homologues are both carbohydrate-binding proteins. MtaL also shares structural homology with the sea mussel galectin CGL from Crenomytilus grayanus (PDB code 5DUY chain A, 8% sequence identity, Z-score of 13.5, r.m.s.d. 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 code 3NBD, chain B (Pohleven et al., 2012)) shows similar residues in the sugar-binding site (Figure 6b). Only two hydroxyl groups, both belonging to the galactosyl moiety of α-lactose, make hydrogen bonds with the CNL protein: the O3 and O4 hydroxyl groups interact with the Asp20 carboxylate, with additional interactions with the Nδ2 of Asn38 (O3), Nδ2 of Asn46 (O3), and the main chain N atom of Gly23 (O4) (Figure 6b, bottom right). No stacking interactions with aromatic side chains are present. Notably, the CNL structure in complex with a non-reducing N-acetylgalactosamine (GalNAc)-containing carbohydrate (LDN, PDB code 3NBE) shows a similar binding to that of lactose, although one additional hydrogen bond is formed between the O2-bound carbonyl oxygen in the acetate group of GalNAc and the hydroxyl oxygen of Ser24 (Pohleven et al., 2012). At the equivalent position in MtaL, sufficient space is available as well for binding a saccharide. Among the residues that could provide hydrogen bonding interactions are Asn24 (equivalent to Asp20 in CNL), Asp42 (His35) and Ser26 (Ser24). Furthermore, loops β2-β3 and β3-β4, which, in CNL, are involved in sugar binding, could have a similar role in MtaL too. In particular, the β3-β4 loop contains a proline residue (Pro46) that must move out of the way to accommodate a sugar molecule, similar to what was observed with its counterpart Pro41 in the CNL-lactose bound structure (Figure 6b, bottom left). Movement of the β3-β4 loop would enable Thr45 (equivalent to Asn38 in CNL) to have hydrogen-bonding interactions with the sugar. Nevertheless, extensive crystal soaking studies to obtain an MtaL structure with a bound carbohydrate (such as lactose, glucose, raffinose, galactose and 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.

Figure 6. (a) Structure-based sequence alignment of MtaL with the ricin-B-like lectin (CNL, PDB code 3NBD, chain B), hemagglutinating protein (HA33, 1YBI, chain B) and the sea mussel lectin (CGL, 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 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 β2-β3 and β3-β4 involved in CNL-sugar binding could also have a similar role in MtaL, where Pro46 in the β3-β4 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 are Asn24 (equivalent to Asp20 in CNL), Asp42 (His35) and Thr45 (Asn38), highlighted in pink and dark blue, respectively.

In conclusion, our findings indicate that MtaL undergoes conformational changes to bind tyrosinase. Whether the proteolytic cleavage of the protruding β2-β3 loop has biological relevance needs further investigation (Espín and 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.