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Published in:
Acta Crystallographica. Section F: Structural Biology and Crystallization Communications Online

Link to article, DOI:
10.1107/S1744309112031004

Publication date:
2012

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):

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Structure of the starch-debranching enzyme barley limit dextrinase reveals homology of the N-terminal domain to CBM21

Barley limit dextrinase (HvLD) is a debranching enzyme from glycoside hydrolase family 13 subfamily 13 (GH13_13) that hydrolyses α-1,6-glucosidic linkages in limit dextrins derived from amylopectin. The structure of HvLD was solved and refined to 1.9 Å resolution. The structure has a glycerol molecule in the active site and is virtually identical to the structures of HvLD in complex with the competitive inhibitors α-cyclodextrin and β-cyclodextrin solved to 2.5 and 2.1 Å resolution, respectively. However, three loops in the N-terminal domain that are shown here to resemble carbohydrate-binding module family 21 were traceable and were included in the present HvLD structure but were too flexible to be traced and included in the structures of the two HvLD-inhibitor complexes.

1. Introduction

Barley limit dextrinase (HvLD) catalyses the debranching of limit dextrins derived from amylopectin, the major constituent of barley starch. Starch amounts to 60% of the total dry weight of cereal grains and consists of an approximately 30:70 mixture of the essentially linear α(1,4)-glucan amylose and the α(1,6)-branched α(1,4)-glucan amylopectin. Enzyme-mediated mobilization of storage starch granules in the endosperm of germinating cereal seeds involves solubilization by the concerted action of α-amylase, β-amylase, limit dextrinase (LD) and α-glucosidase, resulting in dextrins, maltooligosaccharides and glucose. Among these enzymes, only LD has the capacity to hydrolyse α(1,6)-glucosidic linkages in branched α-limit and β-limit dextrins (Kristensen et al., 1999). In addition, LD can hydrolyse α(1,6)-glucosidic linkages in pullulan and, with low efficiency, 1,6-branch points in amylopectin (Kristensen et al., 1999; Burton et al., 1999). LD catalyses hydrolysis via the general acid/base double-displacement mechanism characteristic of glycoside hydrolase family 13 (GH13; Cantarel et al., 2009; MacGregor et al., 2001) by the action of a catalytic nucleophile Asp473 (numbering refers to HvLD; Q9S78) and a catalytic acid/base proton donor Glu510, resulting in retention of the anomeric configuration of the products. Recently, expression of HvLD has successfully been established in Pichia pastoris (Vester-Christensen, Abou Hachem, Naested et al., 2010). The crystal structures of HvLD in complex with the competitive inhibitors α-cyclodextrin (α-CD) and β-cyclodextrin (β-CD) have been solved and refined to 2.5 and 2.1 Å resolution, respectively (Vester-Christensen, Abou Hachem, Svensson et al., 2010). HvLD has four structural domains: the N-domain (residues 1–124), a carbohydrate-binding module from family 48 (CBM48; residues 125–230), the catalytic domain (residues 231–774) and the C-domain (residues 775–885). The structures of HvLD–α-CD and HvLD–β-CD showed overall good electron density, but the two first amino-acid residues and three loops (residues 23–27, 42–48 and 102–109) in the N-domain have low-level or no σA-weighted 2Fo–Fc electron density and were not included in the model (Vester-Christensen, Abou Hachem, Svensson et al., 2010). The function of the N-domain is not clear, but it
is typical of GH13 enzymes that cleave or form endo-α-1,6-linkages (Jespersen et al., 1991) and hence is presumed to have a functional linkage to this specificity. In this paper, we report the 1.9 Å resolution X-ray crystallographic structure of HvLD, including a fully traced backbone of the N-domain which, in spite of a low sequence identity of 6%, can also be seen to possess structural similarity to the N-terminal CBM21 domain of glucoamylase from Rhizopus oryzae (Liu et al., 2007).

2. Materials and methods
2.1. Crystallization, data collection and processing, structure determination and refinement

Recombinant HvLD was prepared using P. pastoris as a host and was purified as described previously (Vester-Christensen, Abou Hachem, Naested et al., 2010). The protein was concentrated to 10 mg ml\(^{-1}\) in 50 mM MES buffer pH 6.6, 250 mM NaCl, 0.5 mM Na\(^{+}\).
In addition to the geometry optimization was performed using the output from CCPet al. molecules and ions were performed with 1992) and scaled with of reflection $R$ of the Refinement 5% glycerol, 0.3 $M$ was added to the crystallization drops to a final concentration of 5–1010 $M$øller (MolProbity score 1.26) dominated by four glycine residues. The resulting structure factors were used for molecular replacement with standard tools such as Refmac5 (Murshudov et al., 2011) and AutoR (Storrie-Burrell et al., 2011). The model was refined using Coot (Emsley et al., 2010). In addition to the Coot validation functions, final analysis of model geometry optimization was performed using the output from PROCHECK and MolProbity (Laskowski et al., 1993; Chen et al., 2010). Two structure-based alignment tools were used in order to advance insight into the possible role of the HvLD N-domain (residues 2–124): a DALLI search (Holm & Rosenstrøm, 2010) against all PDB entries and FATCAT structural alignment (Ye & Godzik, 2003). In addition, a search using PDBBertold (Krisinel & Henrick, 2004) was performed, but no additional information was gained. The structure-based searches were also performed using the N-domain from the HvLD-β-CD structure (PDB entry 2y4s), but the number of significant hits was low compared with the searches with the N-domain from native HvLD owing to the missing loop regions and did not include the CBM21 domain.

3. Results and discussion

3.1. Structure determination and model quality

Two calcium ions, four iodide ions, four glycerol molecules and 294 water molecules were modelled in HvLD. Refinement statistics are listed in Table 1. The geometry of the models is good, with 99.7% of the residues in the allowed regions of the Ramachandran plot and three residues (Lys107, Leu116 and Ala439) in the disallowed region. Ala439 is found in a similar position and intramolecular arrangement as in the HvLD–β-CD structure (PDB entry 2y4s) used for molecular replacement. Lys107 resides in a flexible loop and Leu116 resides in the third α-helix of the N-terminal domain.

3.2. Overall structure

The HvLD structure (Fig. 1a) and the protein moiety of HvLD–β-CD are virtually identical, with an r.m.s.d. of 0.2 Å for all Ca atoms. The major difference between the structures is that the three short loops (residues 23–42 and 102–109) in the N-domain are included in the N-domain of the HvLD structure (Fig. 1b), which consists of seven β-strands arranged in an antiparallel fashion and three α-helices.

Four glycerol molecules (Gol) from the crystallization buffer and the cryoprotectant were found in HvLD (Fig. 1a). Gol1885 is located at the interface between CBM48 and the catalytic domain and Gol1886 is located on the exposed surface of the C-domain. Gol1887 is buried in part of loop 2, similar to Gol306 in HvLD–β-CD. Gol1888 is found in the active site, interacting with the catalytic nucleophile Asp473, and shows the same interaction pattern as a glycerol molecule in HvLD–β-CD (Fig. 1c).

3.3. Active site

The amino-acid residues in the active site of HvLD are found in a similar arrangement and adopt the same rotamers as the amino-acid residues in the HvLD structures with α-CD and β-CD bound in the active site (Fig. 1c).

Mikami et al. (2006) observed a substrate-induced conformational change of the active-site residues connecting the acid/base catalytic residue (Glu706) and the C2 binding site (Tyr70s) in the case of the GH13 pullulanase from Klebsiella pneumoniae, which belongs to the same subfamily as HvLD according to CAZY (Cantarel et al., 2009). They observed two different main-chain conformations of the loop (residues 706–710; EGWDS) depending on whether or not a ligand (in this case glucose, isomaltose, maltose, maltotriose or maltotetraose) was bound. In addition, the side chain of Tyr70s made about a 90° rotation to enable a stacking interaction at the active-site +2 subsite. In the native pullulanase structure (PDB entry 2l2g) and in the structures with bound glucose (PDB entry 2lh8) or isomaltose (PDB entry 2lh8) the loop was in the ‘inactive’ free conformation,
while the loop was in the ‘active’ conformation in the complexes with maltose, maltotriose or maltotetraose (PDB entries 2fhb, 2hnc or 2hfl, respectively; Mikami et al., 2006). The loop is one of the conserved regions of GH13 (MacGregor et al., 2001) and is also conserved in HvLD (residues 510–514; EGWDF). In HvLD the loop is found in the ‘active’ form both in the case of the native structure presented here and in the HvLD–CD complexes, in which the loop and Trp512 in particular participate in binding. Noticeably, Trp512 of native HvLD is also in the ‘active’ rotamer position. It may be argued that the HvLD structure is not in its native state and that the glycerol molecule (Gol1888; Fig. 1c) in the active site could induce the change to the ‘active’ form. However, this does not seem to be a valid explanation since the glycerol molecule is interacting with the catalytic nucleophile Asp473, which is not part of the abovementioned conserved loop that changes conformation and makes no interactions with it. A conformational change upon substrate binding has been observed for several GH13-like enzymes (Barends et al., 2007; Przylas et al., 2000; Hondoh et al., 2003; Woo et al., 2008), among which is a GH13 glucogen-debranching enzyme from *Sulfobolus solfataricus* (Woo et al., 2008), in which the Trp adopts the same rotamer and is in the same position as HvLD when substrate is bound.

These findings suggest that HvLD activity is not dependent on conformational changes of active-site amino-acid residues, unlike the pullulanase discussed above. This may indicate that the active site of HvLD is less flexible, possibly explaining the lower hydrolytic activity of HvLD towards large substrates such as amylopectin and the high activity towards the oligosaccharide limit dextrans compared with bacterial pullulanases.

### 3.4. The N-terminal domain

Superposition of the N-terminal domain of HvLD with those of the deposited HvLD–α-CD and HvLD–β-CD complex structures (Fig. 1b) shows no significant variability in the conformation except for a different tucking in of the N-terminal amino-acid residues 2–5 to the rest of the molecule in the HvLD–α-CD structure (PDB entry 2y5e; Vester-Christensen, Abou Hachem, Svensson et al., 2010) and the previously mentioned well defined loop density of the three flexible loops in the native HvLD structure.

Several alignment methods were explored to advance insight into the possible role of the HvLD N-domain. A DALI search (Holm & Rosenström, 2010) with this domain against the entire PDB archive identified nine unique structures with DALI Z-scores of above 5 (Supplementary Table 1). Only five of these proteins are α-1,6-acting pullulanases belonging to GH13 and the sequence identity to HvLD is in general low (see Supplementary Fig. 1a). Common to the hits is that they, like the HvLD N-domain, do not harbour the active-site residues. Three of the identified domains have documented, albeit diverse, functions. These include binding of a peptide ligand, domain multimerization and N-acetyl-d-glucosamine (NAG) binding (Supplementary Table 1). Noticeably, the parts of the domains involved in these interactions are not structurally similar (Fig. 2). The discrepancy between the amino-acid residues involved in intermolecular interactions and the lack of structural conservation of the same residues indicate that the various functionalities have evolved independently, suggesting that the N-terminal domain is a stable generic scaffold for mediating intermolecular interactions. FATCAT structural alignment (Ye & Godzik, 2003) with the complete N-terminal domain as present in HvLD identified only pullulanase N-terminal domains with a FATCAT P-value of below $1.0 \times 10^{-3}$. Noticeably, the N-terminal starch-binding domain of the CBM21 glucoamylase from *R. oryzae* (PDB entry 2djm; Liu et al., 2007) and the N-domain of HvLD align with a P-value of $1.44 \times 10^{-3}$ despite having a sequence identity of only 6% (Supplementary Fig. 2). Ser76, Tyr78, Ser86 and Lys94 of HvLD are the only surface-exposed residues among the identical residues from the structure-based sequence alignment, and although they are clustered from a steric point of view they are located in a part of the domain which is not structurally conserved (Supplementary Fig. 2).

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1 Supplementary material has been deposited in the IUCr electronic archive (Reference: HV5219).
binding residues identified in *R. oryzae* CBM21 (Tung et al., 2008) are not conserved or are replaced by residues with similar biophysical properties in *Hv*LD (Supplementary Fig. 2). It therefore seems unlikely that these residues play similar roles in the two molecules unless major structural changes occur in *Hv*LD in the presence of starch. In conclusion, the N-terminal domain of *Hv*LD may participate in intermolecular interactions that are important for the *in vivo* functionality of *Hv*LD, but there are no indications of whether the interactions involve multimerization, interactions with other proteins or interactions with substrate.

Access to synchrotron beam time was made possible by support from DANS-CAT. We would like to acknowledge beamline scientist Christoph Mueller-Dieckmann (ESRF beamline ID23-1) for assistance during data collection. MSM was supported by a DTU PhD stipend.

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