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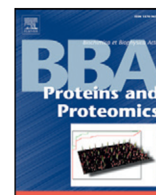

Promoting protein self-association in non-glycosylated *Thermomyces lanuginosus* lipase based on crystal lattice contacts

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ABSTRACT

We have used the crystal structure of *Thermomyces lanuginosus* lipase (TlL) to identify and strengthen potential protein–protein interaction sites in solution. As wildtype we used a deglycosylated mutant of TlL (N33Q). We designed a number of TlL mutants to promote interactions *via* interfaces detected in the crystal-lattice structure, through strengthening of hydrophobic, polar or electrostatic contacts or truncation of sterically blocking residues. We identify a mutant predicted to lead to increased interfacial hydrophobic contacts (N92F) that shows markedly increased self-association properties on native gradient gels. While wildtype TlL mainly forms monomer and <5% dimers, N92F forms stable trimers and dimers according to Size-Exclusion Chromatography and Small-Angle X-ray Scattering. These oligomers account for ~25% of the population and their enzymatic activity is comparable to that of the monomer. Self-association stabilizes TlL against thermal denaturation. Furthermore, the trimer is stable to dilution and requires high concentrations (>2 M) of urea to dissociate. We conclude that crystal lattice contacts are a good starting point for design strategies to promote protein self-association.

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1. Introduction

Lipases are triacylglycerol hydrolases (EC 3.1.1.3) which catalyse hydrolysis of ester groups in triglycerides to produce free fatty acids and glycerol; they also catalyse other reactions involving esters such as transesterification [1]. Their active site contains the catalytic Ser–Asp–His triad known from serine proteases [2]. However, unlike serine proteases, lipases undergo interfacial activation at the lipid–water interface [3], a process in which the lid covering the active site is displaced to make the active site accessible to substrate [4,5]. Due to lipases' versatility they are widely used in industry, in particular within the detergent, food, textile and pulp and paper industries. Particularly used are fungal lipases from *e.g.*, *Candida antarctica*, *Rhizomucor miehei* and *Thermomyces lanuginosus*. The lipase from *T. lanuginosus* (formerly *Humicola lanuginosa*), here designated TlL, consists of 269 residues (after the 17-residue signal peptide and a 5-residue propeptide are removed), and has a natural glycosylation site at Asn33 [6]. Glycosylation does not affect TlL's adsorption on hydrophobic surfaces [3]. Over the past 15 years, TlL has been subjected to many mutations to increase its stability, activity and surfactant tolerance [7,8]. This has highlighted Glu87 and Trp89 in the lid as important side chains for activity [9] as well as stability [10].

The subject of the present work is to use protein engineering to promote intermolecular contacts in TlL. Self-association of lipase has been reported before. The two alkalithermophilic bacterial lipases LipA and LipB naturally form large aggregates in solution. These complexes only dissociate at high concentrations of SDS (~35 mM, *i.e.* well above the critical micelle concentration or cmc) [11]. Lipases from *Pseudomonas fluorescens*, *Candida rugosa*, *Mucor miehie* and *T. lanuginosa* are reported to form dimers at concentrations as low as 0.16 mg/mL; these dimers dissociate in mild detergent (0.5% Triton X-100) and show higher stability but lower specific activity than the monomer [12,13]. This self-association is suggested to occur by mutually induced interfacial activation, so that the hydrophobic region surrounding the active site induces lid-opening and subsequent self-association. However, it is unclear if the effect is partly due to the presence of surfactant micelles. Triton X-100 [14] and other nonionic and ionic surfactants [15] can inhibit TlL activity and can also promote protein association, *e.g.*, by shared micelles [16,17]. Clearly more studies are needed to investigate how TlL can self-associate. Here we exploit intermolecular contact points revealed in different crystal structures of TlL as starting points for the design of a TlL mutant with increased self-association properties, based on the premise that crystal contacts can represent biologically meaningful intermolecular contacts. For added validity, these crystal contacts are then weighted

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through calculation of a complexation significant score. Our starting point is the mutant N33Q which is not glycosylated, hence reducing a potential source of protein heterogeneity. Using this approach, we have identified a TlL mutant (N92F) with significantly increased tendencies to form dimers and trimers. This suggests that crystal contacts can serve as inspiration for design strategies to promote protein self-association.

2. Materials and methods

2.1. Design of TlL N33Q mutants

Crystal structures of TlL were visualized in PyMOL 1.5.0.4 [18] and residues in intermolecular contacts (<5 Å) were identified using PyMol commands. To increase the likelihood of identifying biologically relevant crystal contacts, we used PISA (Protein Interfaces, Surfaces and Assemblies) which calculates a complexation significant score (CSS) for all crystal contacts in a PDB file, based on the structural and chemical properties of the macromolecular surfaces. CSS scores range from 0 to 1. This value is proportional to the probability of the contact representing an interface of a complex that would form under physiological conditions [19]. Mutations of these residues were designed using the following general design principles to promote protein–protein interactions (PPI):

1. Insertion of hydrophobic residues in a hydrophobic area.
2. Insertion of a polar residue to make intermolecular H-bonds or electrostatic interactions.
3. Replacement of a bulky residue with a smaller residue to reduce steric hindrance.
4. Exclusion of water from a crystal contact by increasing hydrophobicity. As a highly polar molecule, water can participate both as donor and acceptor in hydrogen bonds as well as shielding charges in hydrophobic areas. Therefore, excluding water from an interface could both increase and decrease PPI.

2.2. Preparation of mutants

This was carried out by standard site-directed mutagenesis (PCR with mutant oligonucleotides [20]. The inserted TlL gene (based on the gene coding for the 291-residue *T. lanuginosus*, http://www.uniprot.org/uniprot/O59952) omits the 5 amino acid pro-peptide "SPIRR" (residues 18–22) just after the signal peptide and consists of the 17 amino acid signal peptide and the 269 amino acid coding region, together with a N33Q mutation to ensure that TlL remains non-glycosylated (TlL N33Q), to ensure homogenous samples. Mutated plasmids were transformed into *Aspergillus oryzae* protoplasts [21]. *A. oryzae* mutants with the highest TlL expressions were selected based on activity assay with pNP-valerate in microtiter plates and SDS-PAGE [22]. Micro-fermentation and larger-scale fermentation were performed as described [21].

2.3. Micro-purification of TlL mutants

TlL mutants were expressed and purified in small quantities before screening with native-PAGE to find self-associating mutants. The fermentation broth was filtered in a 96 well based micro-filter plate (0.2 μm AcroPrep, PALL) placed on top of a microtiter plate by spinning it in a centrifuge (5810, Eppendorf) at 2000 *g* for 10 min. The TlL mutants were purified from the filtrate by Xpressline ProA8 (Upfront chromatography) loaded onto the 96 well based filterplate (Unifilter 800, Whatman). Prior to TlL binding, the Xpressline ProA was equilibrated twice with an equilibration buffer (1 M Na-acetat, pH 5.0) on a table shaker (MixMate, Eppendorf) at 1000 rpm in 10 min. The unifilter 800 plate was sealed with PCR-thermo tape (Thermo scientific) while incubated on the table shaker. Between each incubation/washing step, the solutions were slowly removed by applying vacuum to a UniVac 3 (Whatman). The filtrate was mixed 3:1 (*v*/*v*) with an equilibration buffer and incubated for 15 min at 1000 rpm with the Expressline ProA beads. This step was repeated until all filtrates had been incubated with the column material. Subsequently, the filter plate was washed with a series of buffers containing 1 M, 0.1 M, 0.01 M and 0.001 M Na-acetate pH 5.0, each with 10 min incubation on the table shaker at 1000 rpm. The filter plate was incubated for 10 min with 10 mM Tris pH 9.0 and eluted directly into a microtiter plate by spinning in a centrifuge (5810, Eppendorf) at 3000 *g* for 10 min. Protein purity was evaluated by SDS-PAGE and compared to SDS-PAGE of the fermentation broth.

2.4. Larger-scale purification

The fermentation broth was filtered in a sterile filter unit (0.45 μm CA polystyrene, Corning) wherein an additional glass microfiber filter (90 mm Ø × 100 circles, Whatman) had been inserted. The filtrate was diluted with 4 M NaCl to obtain a final concentration of 1 M NaCl, before being loaded on an HIC (hydrophobic interaction chromatography) decylamine agarose column that had been equilibrated with 50 mM Tris pH 9.0 and 1 M NaCl. Purification was performed on an Äkta Prime (GE Healthcare) with a flow rate of 10 mL/min and absorbance measured at 280 nm. The column was washed with 2–3 column volumes of 50 mM Tris pH 9.0 and 1 M NaCl and eluted with 50 mM Tris pH 9.0 and 30% isopropanol in fractions of 10 mL. Fractions were then analysed with SDS-PAGE. Those containing TlL were pooled and loaded on an AEC column Q-Sepharose FF (GE Healthcare) equilibrated with 50 mM Tris pH 9.0. The AEC column was washed with 3 column volumes of 50 mM Tris pH 9.0 and eluted with a 0–100% linear gradient of 50 mM Tris pH 9.0 and 1 M NaCl (increasing 1% per 20 s.). The eluent was collected in 10 mL fractions and analysed by SDS-PAGE.

2.5. Native-gel electrophoresis

We used CN-PAGE (Clear/Colourless-Native Polyacrylamide Gel Electrophoresis), in which the protein migrates by virtue of its intrinsic negative charge at pH 8.3, without the need to add anions such as Coomassie Brilliant Blue. At this pH, TlL (pI *ca.* 4.9) has a charge of around −10. TlL mutants with charges between +1 and +7 compared to N33Q TlL (*i.e.* net charges of −9 to −3) were also run as charge markers. Native-PAGE gradient gels with either 4–15% or 20% polyacrylamide were purchased from Biorad (Criterion Tris–HCL precast gels). Samples were mixed with a sample buffer to a final concentration of 31 mM Tris–HCl pH 6.8 and 20% (*v*/*v*) glycerol. A running buffer with 192 mM glycine in 25 mM Tris pH 8.3 was used in both the upper and lower buffer chambers. The gels were run at 4 °C with a pre-cooled running buffer (4 °C) at 200 V and 50 mA for 1 h. After the run, gels were stained 1 h with Imperial protein stain (Thermo Scientific) to visualize protein bands and scanned on a HP Scanjet 4890 printer. Densitometric analysis was carried out with ImageJ 1.50b (Wayne Rasband, NIH). Oligomer stability towards 5 mM of nonionic detergents (dodecyl maltoside, Tween-20) and 5 mg/mL olive oil were tested with 0.2 mg/mL V17 on CN-PAGE using the conditions as described.

2.6. Size-exclusion chromatography

A Superdex 200 10/300 GL (GE Healthcare) column was run on an Äkta Prime system. The column was calibrated with a set of standard proteins (12.4 kDa cytochrome C, 29 kDa carbonic anhydrase, 44 kDa ovalbumin, 66 kDa BSA, 75 kDa Conalbumin and 158 kDa aldolase).

2.7. Circular dichroism

The mutant V17 was purified by SEC into fractions representing the monomeric and oligomeric species, resulting in a monomeric, mixed

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