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Research paper

Supported inhibitor for fishing lipases in complex biological media and mass spectrometry identification

Vincent Delorme [a, b], Brigitt Raux [a], Rémy Puppo [c], Julien Leclaire [b, *], Jean-François Cavalier [a], Sylvain Marc [b], Pavan-Kumar Kamarajugadda [b], Gérard Buono [b], Frédéric Fotiadu [b], Stéphane Canaan [a, **], Frédéric Carrière [a]

[a] CNRS, Aix-Marseille Université, Enzymologie Interfaciale et Physiologie de la Lipolyse, UMR 7282, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France
[b] CNRS, Aix Marseille Université, Centrale Marseille, iSm2 UMR 7313, avenue Escadrille Normandie-Niemen, 13397 Marseille Cedex 20, France
[c] CNRS, Aix Marseille Université, Institut de Microbiologie de la Méditerranée FR3479, plate-forme Marseille Protéomique (MaP), 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France

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ABSTRACT

A synthetic phosphonate inhibitor designed for lipase inhibition but displaying a broader range of activity was covalently immobilized on a solid support to generate a function-directed tool targeting serine hydrolases. To achieve this goal, straightforward and reliable analytical techniques were developed, allowing the monitoring of the solid support's chemical functionalization, enzyme capture processes and physisorption artifacts. This grafted inhibitor was tested on pure lipases and serine proteases from various origins, and assayed for the selective capture of lipases from several complex biological extracts. The direct identification of captured enzymes by mass spectrometry brought the proof of concept on the efficiency of this supported covalent inhibitor. The features and limitations of this "enzyme-fishing" proteomic tool provide new insight on solid–liquid inhibition process.

1. Introduction

Protein class-directed chemical probes deserve considerable attention as innovative technologies in chemoproteomics [1,2]. In this context, the optimized design of immobilized inhibitor provides access to activity-based probes (ABP) that can serve as specific capture agents for enzymes. The main advantage of such ABPs is that they can be used on complex biological material to selectively sequester target proteins with minimal sample preparation and processing. Such a technology can thereby be used for preparative purposes in affinity chromatography or for the identification of so-far unknown biomacromolecular targets [3–5]. In addition, this method can inform on the degree of selectivity of the grafted molecule towards various enzymes within the medium investigated [1,2]. In various biological contexts, grafted inhibitors serve to considerably improve comparative proteomic analyses, *i.e.* to monitor phenotypic changes upon exposure to chemical or biological stimuli [6]. Two alternative approaches can be envisaged for the function-directed capture of enzymes: the first one consists in the incubation of soluble inhibitors followed by the capture of the enzyme–inhibitor complex by a functionalized solid matrix. The second involves the preliminary immobilization of the inhibitor on the support and a subsequent solid/liquid inhibition-capture process [7].

Within the hydrolytic enzyme family, serine and cysteine hydrolases, including proteases, lipases and carboxylesterases, are the most abundant classes of enzymes in the living world [8]. In particular, lipases represent a specific class of carboxylesterases hydrolyzing insoluble triacylglycerol substrates and play key roles in fat metabolism, energy mobilization, and bacterial growth.

Abbreviations: ABP, activity-based probes; AChE, acetylcholinesterase; BSA, bovine serum albumin; DGL, dog gastric lipase; ESI-Q-ToF, electrospray quadrupole time of flight; FM, foamy macrophage; *h*CEH, human pancreatic carboxylester hydrolase; HPL, human pancreatic lipase; ILI, intracellular lipidic inclusion; LB, lipid body; PMF, peptide mass fingerprint; PLGS, protein lynx global server software; PL-PEGA, poly[acryloyl-bis(aminopropyl)polyethylene glycol]; *p*NP, *para*-nitrophenol; *p*NPP, *para*-nitrophenyl phosphonate; PPD, protein purified derivative; TAG, triacylglycerol; TB, tuberculosis.

* Corresponding author. Current address: CNRS, Université Claude Bernard Lyon 1, CPE Lyon, ICBMS UMR 5246, 43 bd du 11 Novembre 1918, 69622 Villeurbanne Cedex, France. Tel.: +33 426234404.
** Corresponding author. Tel.: +33 491164093; fax: +33 491715857.
E-mail addresses: julien.leclaire@univ-lyon1.fr (J. Leclaire), canaan@imm.cnrs.fr (S. Canaan).

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Reactive *para*-nitrophenyl phosphonate (*p*NPP)-based inhibitors revealed as relevant probes, targeting the active site of such hydrolases by forming an irreversible covalent bond with the catalytic residue [9–12]. More generally, phosphonate inhibitors are amongst the most often used probes to efficiently capture lipolytic enzymes or proteases in solution, followed by solid capture of the resulting complex [13–15]. The alternative strategy of direct solid–liquid capture was envisaged during pioneering studies conducted in the 1970s using immobilized *p*NPP in order to purify acetylcholinesterase (AChE) from biological extracts [16]. Such systems were subsequently synthetically revisited and applied to the selective removal of chymotrypsin-like proteases from biological samples using diphenyl α-aminoalkylphosphonate immobilized on sepharose gel [17]. We have chosen to evaluate the potency and limitations of this second option, as we were particularly interested in the physical and chemical features of interfacial, heterogeneous and covalent inhibition process of lipases and carboxylesterases using *p*NPP-based probes.

In the perspective of exploring the strength and limitations of covalent solid–liquid inhibition, we tried to minimize the synthetic and analytical investment required to build and use the grafted inhibitor. One of the main difficulties of solid–liquid covalent capture relies in the analysis of both the supported inhibitor and immobilized protein. Early reports by Reetz et al. [12] suggested the need for routinely accessible analytical techniques to monitor the chemical construction of this ABP and assess the effective amount of available supported active species. The design of a reliable, yet accessible set of analytical assays is therefore crucial to envisage the use of such a molecular architecture for the capture of proteins from complex biological mixtures. The chemical immobilization of the inhibitor on the solid support is far from being a trivial issue either [18]. An additional challenge while targeting lipolytic enzymes comes from the specific nature of the enzyme activation, which is sometimes related to conformational changes involving the opening of a lid that provides access to the active site and can be triggered by the presence of lipids or amphiphiles [19,20]. Molecular elements that can induce this conformational transition toward the active form prior to capture must therefore be implemented in the design either of the immobilized grafted inhibitor (by selecting an amphiphilic inhibitor whose profile shall be preserved after grafting) or of the assay (by the use of amphiphilic auxiliaries in the incubation medium).

The objective of this study consisted in developing, characterizing and testing on complex mixtures such as entire cellular media or digestive fluids, a molecular tool directed toward serine hydrolases and more specifically lipases and carboxylesterases.

2. Materials and methods

2.1. Solid support

Poly[acryloyl-bis(aminopropyl)polyethylene glycol] (PL-PEGA) resins, reagents and chemicals were purchased from Sigma–Aldrich–Fluka Chimie (St-Quentin-Fallavier, France). The methods describing the synthesis and chemical characterization of the compounds **1** to **5** are available in Supporting information.

2.2. Purified enzymes

Fusarium solani (*Fs*) cutinase, human pancreatic lipase (HPL), dog gastric lipase (DGL) and LipY lipase from *Mycobacterium tuberculosis* were produced as recombinant enzymes and purified following previously described procedures [21–24]. Native human pancreatic carboxylester hydrolase (*h*CEH) was purified to homogeneity from human pancreatic juices according to the method previously described [25] and was kindly provided by Dr S. Amara. Trypsin from bovine pancreas was purchased as a lyophilized powder from Sigma–Aldrich (ref T9935).

2.3. Biological media

Materials for cell growth and protein expression were from Sigma–Aldrich, unless otherwise noted.

2.3.1. Surexpression of Fs Cutinase [21]

Crude samples containing *Fs* Cutinase were prepared from cell cultures of *Escherichia coli* BL21 (DE3) (Invitrogen) carrying pFCEX1D plasmid. Cells were grown at 25 °C in Luria Broth (LB) medium (Life Technologies) containing ampicillin (100 μg/mL). Induction was performed at OD_{600} of 1.5, using 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 7 h. Cells were then harvested by centrifugation at 5000× *g* for 10 min at 4 °C and resuspended in ice-cold lysis buffer (50 mM Tris–HCl, pH 8.0 containing 150 mM NaCl, 0.1% Triton X-100, and 0.25 mg/mL lysozyme, 30 mL/L of initial culture). DNAse (final concentration 10 μg/mL) and magnesium sulfate (final concentration 20 mM) were added and mixture was incubated for 20 min at room temperature. Mixture (10 mg/mL total proteins) was then freshly used as crude sample for capture experiments.

2.3.2. Surexpression of LipY [26]

Crude samples containing LipY were prepared from cell cultures of *Mycobacterium smegmatis* mc^2155 carrying recombinant pSD26 plasmid containing the *LipY* gene. Cells were grown at 37 °C in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween-80 (v/v), 0.2% glycerol (v/v), 0.5% bovine serum albumin (BSA) (w/v), 0.2% glucose (w/v) and 50 μg/mL hygromycine B. Induction was performed at OD_{600} of 3.0, using acetamide (0.2%, w/v) for 16 h. Cells were then harvested by centrifugation at 5000× *g* for 10 min at 4 °C and resuspended in ice-cold 10 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl and 1% *N*-lauroylsarcosine. Cells were then passed twice through a French Press set at 1100 psi. Homogenate (10 mg/mL total proteins) was then freshly used as crude sample for capture experiments.

2.3.3. Human pancreatic juice

Crude pancreatic juice samples were collected from healthy volunteers as previously described [27], lyophilized and stored at −20 °C. 10.0 mg of dry powder was dissolved in 1 mL of 10 mM Tris–HCl buffer (pH 7.5) containing 500 mM NaCl in order to reconstitute the pancreatic juices. When needed, protease inhibitors cocktail (complete Mini EDTA-free, Roche Diagnostic GmbH) was also added (0.1%, w/v), complemented with phenylmethylsulfonyl fluoride (PMSF, 1 mM) and Benzamidine (2 mM). The mixture (10 mg/mL of total proteins corresponding to 0.2 mg/mL of HPL) was stirred for 1 h at 4 °C, centrifuged and the supernatant was immediately used as a crude sample for capture experiments.

2.4. Synthesis and analysis of grafted inhibitors

2.4.1. Method for the grafting of PL-PEGA with 5 (adapted from [12])

300 mg (0.12 mmol) of dry PL-PEGA resin was washed 3 times with 5 mL of dry acetonitrile. The resin was dried under high vacuum and swelled in a solution of **5a** or **5b** (0.6 mmol) and triethylamine (121 mg, 1.2 mmol) in dry acetonitrile (15 mL). The reaction mixture was mechanically stirred using a thermomixer unit for 2 days at room temperature. Immobilized inhibitors **6a** and **6b** were further washed with acetonitrile (3 × 10 mL), DCM