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Stabilisation and characterisation of the isolated regulatory domain of human 5-lipoxygenase

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ABSTRACT

5-Lipoxygenase (5-LOX) is the key player of pro-inflammatory leukotriene biosynthesis. Its regulatory or so-called PLAT (polycystin-1, lipoxygenase, α-toxin) domain binds allosteric modulators like calcium, membranes, coactosin-like protein and Dicer, thereby influencing 5-LOX activity at the nuclear membrane by mediating translocation. The PLAT domain may also regulate cytosolic 5-LOX activity and possibly influence microRNA metabolism. Hence, it has also evolved as a promising target for anti-inflammatory therapy. Research focusing on this substructure of 5-LOX requires an assay system based on the isolated domain. However, we found that the isolated PLAT domain was highly prone to aggregation and therefore unsuitable for interaction studies. Substitution of the single, membrane-binding tryptophan 75 with glycine reduced aggregation and substantially increased its thermal stability. Calcium interaction of the single mutant was confirmed by differential scanning fluorimetry. Moreover, crosslinking experiments demonstrated the ability of the isolated PLAT domain to bind Dicer C-terminus whereas the interaction with coactosin-like protein required the interplay of the catalytic and the PLAT domain.

1. Introduction

5-Lipoxygenase (5-LOX) (for review see [1,2]) converts arachidonic acid in a two-step reaction into leukotriene A_4 (LTA_4). This intermediate can be further metabolised to yield the full spectrum of pro-inflammatory leukotrienes that play an important role in normal host defence and inflammatory diseases like in asthma [3], atherosclerosis [4] and various types of cancer [5–7]. Despite extensive efforts in drug discovery, up to now only one inhibitor, zileuton, obtained market authorisation (for review see [8]). Therefore, further research on 5-LOX regulation and the development of novel strategies for 5-LOX inhibition are highly needed. The structure of 5-LOX exhibits two distinct domains, the regulatory so-called PLAT (polycystin-1, lipoxygenase, α-toxin) domain (aa 1–115) and the catalytic domain (aa 121–673), which occupies the non-haem iron in the catalytic centre. Targeting the PLAT domain constitutes a novel, promising inhibitory mechanism [9,10]. However, the identification of the corresponding inhibitors is laborious in case that studies are conducted with the full-length enzyme. Consequently, for the development of efficient screening techniques a stable, isolated PLAT domain is required.

In 2011, Gilbert et al. succeeded in the construction of a stabilised 5-LOX mutant (Stable 5-LOX) and the elucidation of its crystal structure (PDB ID: 3O8Y [11]). Even though the PLAT domain is heavily mutated in its calcium and membrane binding sites, their studies confirm what had been derived from the structures of e.g. rabbit reticulocyte 15-LOX (2SBL [12]) and *Clostridium perfringens* α-toxin (1QMD [13]): the regulatory domain adopts a C2-like structure which is characterised by two sheets, each containing four antiparallel β-strands connected by flexible loops. The latter ones (so-called loops 1, 2, 3 and 4 [14]) determine binding of membranes [15], calcium [14], coactosin-like protein (CLP) [16], Dicer [17] and several other factors essentially affecting 5-LOX activity [9,18,19]. Until now, most investigators elucidated the regulatory impact of the aforementioned allosteric modulators on the full-length enzyme. But due to the fact that arachidonic acid [20] as well as ATP [18] may bind to both domains, some questions cannot fully be

Abbreviations: βME, β-mercaptoethanol; BS3, bis[sulfosuccinimidyl]suberate; BSA, bovine serum albumin; CLP, coactosin-like protein; cv, column volume; DF, Dicer fragment containing amino acids 1650–1912; DPBS, Dulbecco's PBS; DSF, differential scanning fluorimetry; EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide; Ig-CA, Igepal® CA-630; IPTG, isopropyl β-D-thiogalacto-pyranoside; LOX, lipoxygenase; LTA_4, leukotriene A_4; MBP, maltose-binding protein; PC, phosphatidylcholine; PLAT, polycystin-1, lipoxygenase, α-toxin; SEC-MALS, size exclusion chromatography coupled with multi-angle light scattering detection; s-NHS, N-hydroxysulfosuccinimide; T20, Tween 20; TEV, tobacco etch virus protease; T_m, melting temperature; T_0, reference melting temperature without additive; ΔT_m, change in melting temperature compared to reference melting temperature

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answered by addressing full-length 5-LOX. Thus, studies on the isolated PLAT domain provide additional knowledge on 5-LOX allosteric regulation and therefore also the regulation of leukotriene biosynthesis.

5-LOX is regulated in a highly complex manner. Upon stimulation of the cell and subsequent calcium release, it translocates to the nuclear envelope and perinuclear membranes [21] in order to gain access to arachidonic acid. In vitro, 5-LOX reaches maximal activity at 4–10 μM Ca^{2+} [22–24]. Besides, equilibrium dialysis revealed a 2:1 stoichiometry (Ca^{2+}: 5-LOX) with an apparent K_D of 6 μM [25]. The isolated, hexahistidine-tagged PLAT domain shows comparable affinity [15]. Mutagenesis of several charged residues in loop 2 (N43/D44/E46A) impairs calcium binding and subsequently shifts maximal 5-LOX activation up to 100 μM Ca^{2+} [14]. In addition to 5-LOX translocation, calcium mediates its association with phosphatidylcholine (PC) liposomes in vitro [23]. Mutation W13A, W75A or W102A of the isolated PLAT domain reduces its binding affinity and specificity for PC with W102A showing the strongest effect [15]. Concerning the full-length enzyme, the influence of W13/75/102A with respect to 5-LOX activity is only visible at low PC and arachidonic acid concentrations [16,26].

CLP, an F-actin binding protein [27], is another important interaction partner. It stabilises 5-LOX [16] and alters its activity in the presence and absence of PC [26]. Their direct interaction was demonstrated e.g. by GST-pulldown [16,26,28], crosslinking [28] as well as on the 5-LOX activity level [16,26]. Interestingly, binding itself was shown to be calcium-independent whilst activation of 5-LOX requires the presence of either calcium or magnesium [26,28]. Besides, substitution of W102 interrupts all effects of CLP coincubation, stressing the importance of the PLAT domain [16].

5-LOX activity can also be altered by Dicer, which plays an important role in microRNA and small interfering RNA metabolism [29,30]. Dicer is a multi-domain protein of the RNase III family. Its connection to 5-LOX was discovered when partial cDNA clones of its C-terminus were selected from yeast two-hybrid system [31]. The effect of binding seems to be two-directional: on the one hand the C-terminal fragment of Dicer enhances 5-LOX activity; on the other hand 5-LOX alters in vitro pre-microRNA processing of full-length Dicer [17]. Dincbas-Renqvist et al. suggested the involvement of the PLAT domain because 5-LOX W13/75/102A is unable to bind the Dicer C-terminus in a GST-pulldown assay in contrast to the wild-type protein [17].

In this study, we report both the construction and characterisation of a monomeric, thermally stabilised PLAT domain mutant, 5-LOX PLAT W75G. Detailed analysis was performed focussing on calcium binding and the interaction with Dicer and CLP. We also present a new test system based on differential scanning fluorimetry (DSF) now enabling elaborate studies to further clarify the role of this regulatory domain for 5-LOX activity and pro-inflammatory leukotriene production.

2. Materials and methods

2.1. Materials

Materials were purchased from the following sources: Bio-Rad, Hercules, USA (BT Chelex® 100 resin, Bradford reagent, Precision Plus Protein™ Standard all blue); GE Healthcare, Little Chalfont, England (HisTrap HP 1 ml, HiPrep Desalting 5 ml, Resource Q 1 ml, HiPrep 16/60 Sephacryl S-200 HR and Superdex 200 10/300 GL, Glutathione Sepharose 4B, high molecular weight gel filtration calibration kit, Hybond-C extra nitrocellulose membrane, Dulbecco's PBS (DPBS)); Life Technologies Corporation, Carlsbad, USA (LB medium, SYPRO® orange); LI-COR Biosciences, Lincoln, USA (secondary antibodies and blocking buffer); Merck Millipore, Darmstadt, Germany (*Escherichia coli* Rosetta™ 2(DE3)); New England Biolabs, Ipswich, USA (amylose resin high flow, *E. coli* BL21(DE3), restriction enzymes); Proteintech, Chicago, USA (anti-CLP COTL1 antibody); Qiagen, Hilden, Germany (factor Xa protease); Santa Cruz Biotechnology, Dallas, USA (anti-5-LOX N-19 antibody, anti-Dicer H-212 antibody); Sigma-Aldrich, St. Louis, USA (adenosine 5′-triphosphate-agarose, kit for molecular weights 6.5–66 kDa, anti-bovine serum albumin (BSA) antibody from rabbit, calcium atomic spectroscopy standard); Stratagene, Santa Clara, USA (*E. coli* SURE); Thermo Fisher Scientific, Waltham, USA (bis[sulfosuccinimidyl] suberate (BS3), 1-ethyl-3[3-dimethylamino-propyl]carbodiimide (EDC), N-hydroxysulfosuccinimide (s-NHS)), produced in-house (anti-5-LOX 6A12 antibody).

All remaining chemicals were obtained from AppliChem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Sigma-Aldrich.

Oligonucleotides were synthesised by Eurofins MWG Operon (Ebersberg, Germany) and sequencing of PCR products was performed by Scientific Research and Development GmbH (Oberursel, Germany).

The following vectors were used for cloning or recombinant protein expression: pBUF1b; pNIC-28-Bsa1-Dicer1650-1912 [17]; pGEX-5X-1-CLP [28]; pT3-5-LOX [32]; pET14b-Stable-5-LOX [11] (kindly provided by Prof. M. E. Newcomer, Louisiana State University, Baton Rouge, USA).

2.2. Methods

2.2.1. Cloning of PLAT expression vectors

DNA encoding the PLAT domain (aa 1–115 of human 5-LOX) or the so-called Soluble PLAT domain (5-LOX1–115 W13E/F14H/Δ40–44GS/W75G/L76S) [11] was amplified from pT3-5-LOX or pET14b-Stable-5-LOX, respectively, by PCR (see Supplementary material S1 for primer sequence). The PCR product was cleaved with EcoR1 and Sal1 and cloned into the expression vector pBUF1b. Insertion of the fragment was confirmed by DNA sequencing. The encoded PLAT fusion protein [33] consisted of maltose-binding protein (MBP) followed by a hexahistidine-tag, tobacco etch virus protease (TEV) cleavage site and aa 1–115 of human 5-LOX or 5-LOX mutant. The isolated PLAT domain derived from TEV cleavage of the corresponding fusion protein is hereafter referred to as PLAT.

Point mutations for generation of PLAT Δ40–44GS/W75G and PLAT W75G expression vectors were introduced via circular site-directed mutagenesis based on the principles of the QuickChange™ Site-Directed Mutagenesis System by Stratagene [34] (see Supplementary material S1 for primer sequence). The PCR product was transformed into *E. coli* SURE for ligation and multiplication. Mutations were verified by sequence analysis.

2.2.2. Purification of PLAT

The PLAT fusion protein was expressed in *E. coli* BL21(DE3) and purified on an amylose resin high flow column as described elsewhere [33]. The eluate in 20 mM Tris, 10 mM maltose pH 7.5 was diluted to a protein concentration less than 2 mg/ml and cleaved with TEV at 4 °C over night or at 18 °C for 1 h. After cleavage, the pH was increased with 20 mM triethanolamine pH 8.0 and the salt concentration was adjusted to 0.3 M NaCl. Next, the PLAT was applied to a HisTrap HP column in 20 mM Tris, 0.3 M NaCl pH 8.0. Elution fractions at 28 mM imidazole were pooled and supplied with 1 mM EDTA and 1–10 mM DTT.

Expression, purification and TEV cleavage of the PLAT mutant fusion proteins were performed as described for PLAT. The cleavage products were applied to a HisTrap HP column at pH 7.4 without further adjustments. PLAT mutants eluted with the column flow through. After addition of 1 mM EDTA and 10 mM DTT corresponding fractions were concentrated on an Amicon® stirred cell (Merck Millipore) and run on a HiPrep Sephacryl S-200 column in DPBS, 1 mM EDTA, 5 mM DTT pH 7.4.

In general, all steps were carefully monitored to ensure temperatures below 20 °C. Due to the purification strategy, all cleaved PLAT constructs contain the additional peptide GSEF at the N-terminus. Assays were conducted in the following two days.

2.2.3. Purification of 5-LOX

E. coli BL21(DE3) transformed with pT3-5-LOX was grown for 5 h at 37 °C in LB and 5-LOX expressed over night at 22 °C after induction with