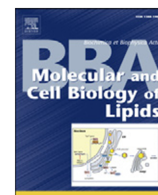
Biochimica et Biophysica Acta 1851 (2015) 1465–1472

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip



Characterization of the interaction of human 5-lipoxygenase with its activating protein FLAP

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ARTICLE INFO

Article history:
Received 28 March 2015
Received in revised form 10 August 2015
Accepted 26 August 2015
Available online 29 August 2015

Keywords:
Leukotrienes
Inflammation
Lipoxygenase
Subcellular Localization
FLAP

ABSTRACT

Human 5-lipoxygenase (5-LO) is the key enzyme in the formation of leukotrienes (LTs), important mediators of inflammation. Cellular 5-LO activity is regulated in a complex manner, e.g. by calcium influx, the cellular redox status or 5-LO phosphorylation. Being a mobile enzyme, 5-LO migrates from the cytosol to the nuclear envelope where it is believed to interact with 5-lipoxygenase-activating protein (FLAP) and receives the substrate arachidonic acid (AA). 5-LO contains four cysteine residues located close to the AA entry site. In the present study, we show that in vitro glutathionylation of recombinant purified 5-LO wildtype (WT) as well as 5-LO 4C, a mutant where the four surface cysteines are replaced by serines (Cys159/300/416/418Ser), does not alter the product synthesis. However, in 5-LO/FLAP-transfected HeLa cells, treatment with the thiol-oxidizing agent diamide which promotes glutathionylation at surface Cys residues led to a decreased LT synthesis by 5-LO WT. In contrast to the WT enzyme, LT formation of the 4C mutant was stimulated by addition of diamide. Immunofluorescence studies in human monocytes and HEK293 cells, expressing 5-LO and FLAP, revealed that diamide prevented the translocation of 5-LO WT whereas it enhanced the translocation of the fourfold cysteine mutant. Therefore, we could demonstrate that the interface, involving the four cysteines 159, 300, 416 and 418, is important for the translocation to the nuclear membrane and the colocalization with FLAP.

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1. Introduction

5-Lipoxygenase (5-LO) catalyzes the first step in the formation of leukotrienes (LTs). LTs are important mediators of inflammation and play a crucial role in many diseases like asthma [1,2] and atherosclerosis [3,4]. A participation of the 5-LO pathway in the development of different types of cancer like leukemia [5], pancreatic [6,7] or colorectal cancer [8] has also been suggested.

5-LO catalyzes two initial steps in the metabolism of arachidonic acid (AA), first the oxygenation of AA to 5(S)-hydroperoxy-6,8,11,14-(E,Z,Z,Z)-eicosatetraenoic acid (5-HpETE), followed by the dehydration to the instable epoxide leukotriene A_4 (LTA_4). This intermediate can be converted either by LTA_4 hydrolase to leukotriene B_4 (LTB_4) or by LTC_4 synthase to the cysteinyl leukotriene C_4 that is subsequently cleaved to LTD_4 and LTE_4.

Human 5-LO consists of 673 amino acids (aa). Recently, the X-ray crystal structure of a 5-LO mutant, so-called Stable-5LOX, was determined [9]. It can be divided in two domains, a smaller N-terminal regulatory C2-like domain (C2ld, aa 1–115) and the larger C-terminal catalytic domain (aa 121–673). The C2ld is responsible for binding of calcium (Asn43, Asp44 and Glu46) [10], membranes (Trp13, Trp75 and Trp102) [11], the interaction with coactosin-like protein [12] and dicer [13] and mainly consists of β-sheets. The α-helical C-terminal domain contains the catalytic center with the non-heme iron, coordinated by His367, His372, His550, Asn554 and the C-terminus Ile673 [14,15]. Moreover, 5-LO has two ATP-binding sites, one located in the C2ld (aa 73–83), the other in the catalytic domain (aa 193–209) [16].

In the cell, 5-LO can be located either in the cytosol or in the nucleus, depending on the cell type. After calcium binding and phosphorylation, 5-LO is activated and migrates to the nuclear membrane where it colocalizes with the cytosolic phospholipase A_2 (cPLA2) that releases AA from the phospholipids and with the 5-lipoxygenase-activating protein (FLAP) that is thought to transfer AA to 5-LO. FLAP, an 18 kDa integral nuclear membrane protein, is a member of the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) family (for review see [17]). Unlike other proteins of the MAPEG family, it

Abbreviations: 5-H(p)ETE, 5S-hydro(pero)xy-6,8,11,14(E,Z,Z,Z)-eicosatetraenoic acid; 6-trans-LTB_4, 5S,12R-dihydroxy-6,8,10,14(E,E,E,Z)-eicosatetraenoic acid; 6-trans-12-epi-LTB_4, 5S,12S-dihydroxy-6,8,10,14-(E,E,E,Z)-eicosatetraenoic acid; Aa, amino acid; AA, arachidonic acid; DAPI, diamidino-2-phenylindole; FLAP, 5-lipoxygenase-activating protein; GSH, reduced glutathione; LO, lipoxygenase; LT, leukotriene; LTB_4, leukotriene B_4 (5S,12R-dihydroxy-6,8,10,14(Z,E,E,Z)-eicosatetraenoic acid); PBS, Dulbecco's phosphate-buffered saline; PC, phosphatidylcholine; WT, wildtype.

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http://dx.doi.org/10.1016/j.bbalip.2015.08.010
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does not possess any catalytic activity. However, treatment with MK886, a direct FLAP inhibitor, blocks the formation of 5-LO products [18,19]. In 2007, the structure of inhibitor-bound FLAP could be resolved as a trimer where each monomer contains four transmembrane helices [20]. But despite the fact that the structures of 5-LO and FLAP are known, the direct mechanism how FLAP provides 5-LO with AA and supports LT formation is only partly understood and requires further investigations.

Recently, we could show that 5-LO is able to form dimers [21] and we identified four cysteines (Cys159, Cys300, Cys416 and Cys418) located in the dimerization interface that comprises the region around the entrance of the catalytic center. Modification of 5-LO with glutathione (GSH) by GSH/diamide treatment blocked dimerization. Mutation of the cysteines to serines prevented the formation of dimers crosslinked via disulfide bonds. Additionally, we demonstrated that inhibition of 5-LO activity by the well-known phospholipase C inhibitor U73122 is due to covalent binding to cysteine 416 [22].

As 5-LO is prone to redox regulation in the cell, e.g. 5-LO inhibition by glutathione peroxidase (GPX) 1 and 4 [23–26], localization and activity of 5-LO and its mutants 5-LO 3W (Trp13,75,102Ala) and 5-LO 4C (Cys159,300,416,418Ser) were investigated, applying a model of increased oxidative cellular stress by diamide treatment that leads to an increased S-glutathionylation of intracellular proteins [27,28].

2. Materials and methods

2.1. Materials

Frankfurt: AA, calcium ionophore A23187, diamide, ATP-agarose, BWA4C, GSH, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (PC), MK886 and penicillin/streptomycin were purchased from Sigma Aldrich (Taufkirchen, Germany). Fetal calf serum (FCS) was purchased from Biochrom AG (Berlin, Germany), and Dulbecco's modified Eagle's medium (DMEM) from Gibco (Life Technologies, Darmstadt, Germany). HPLC solvents were obtained from Merck (Darmstadt, Germany). HeLa cells (no: ACC57) were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH) and the plasmid pT3-5-LO from Dr. Olof Rådmark (Stockholm, Sweden).

Jena: DMEM, FCS, LSM 1077, penicillin, RPMI, streptomycin, trypsin/EDTA and geneticin were from PAA Laboratories (Coelbe, Germany). Lipofectamine LTX Reagent Plus, 10% non-immune goat serum, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 555 goat anti-mouse, diamidino-2-phenylindole (DAPI) and hygromycin B were from Invitrogen (Darmstadt, Germany). The mouse anti-5-LO monoclonal antibody was produced in-house at Goethe-University, Frankfurt, Germany. The rabbit anti-FLAP polyclonal antibody was from Abcam (Cambridge, UK). The FLAP inhibitor MK886 was from Cayman Chemicals (AnnArbor, US). Calcium ionophore A23187, Triton X-100 and diamide were from Sigma Aldrich.

2.2. Methods

2.2.1. Cells

Jena: Peripheral blood mononuclear cells (PBMC), containing human primary monocytes and lymphocytes were freshly isolated from leukocyte concentrates obtained from the Institute of Transfusion Medicine, University Hospital Jena, as described [29]. In brief, PBMC were isolated by dextran sedimentation and centrifugation on lymphocyte separation medium (LSM 1077, PAA, Coelbe, Germany). Cells were seeded onto glass coverslips for 1 h at 37 °C for adherence in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin, before subjection to subcellular localization analysis by immunofluorescence microscopy.

Frankfurt: Human leukocytes were freshly isolated from leukocyte concentrates obtained from the Deutsche Blutspendedienst in Frankfurt. Each leukocyte concentrate (obtained from 500 ml peripheral blood, each) was collected and diluted with Dulbecco's phosphate-buffered saline pH 7.4 (PBS). Dextran sedimentation was carried out by the addition of 10 ml 5% dextran solution to 40 ml of the diluted leukocyte concentrates. After 30–40 min of sedimentation, the supernatants were collected and subjected to density gradient centrifugation. 40 ml of the cell suspension was layered on top of 10 ml of Nycoprep 1.077 solution and centrifuged at 800 ×g for 10 min. The mononuclear cells were collected and diluted with PBS with Ca^{2+}, pH 7.4, centrifuged (300 ×g, 10 min) and washed three times with PBS with Ca^{2+}, pH 7.4. The cell pellets were taken up in serum-free RPMI 1640 medium and cells were grown for 1 h under standard culture conditions. Then, the supernatant was removed and the adherent monocytes were washed three times with PBS without Ca^{2+} and were finally taken up in PBS pH 7.4 containing 1 mg/ml glucose.

2.2.2. Cell culture

HeLa and HEK293 cells were cultured at 37 °C and 5% CO_2 in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. HEK293 cell lines stably expressing 5-LO as well as 5-LO mutants with or without FLAP were selected using 400 μg/ml geneticin and/or 200 μg/ml hygromycin B, respectively [30].

2.2.3. Transient transfection

Plasmid DNA (25 μg of pcDNA3.1-5LO, pcDNA3.1-3W or pcDNA3.1-4C, and/or 10 μg pSG5-FLAP) was transiently transfected into HeLa cells using the calcium phosphate method [31] cultured for 48 h and afterwards assayed for 5-LO activity.

2.2.4. Expression and purification of human 5-LO

Recombinant 5-LO WT or the mutants 5-LO 3W [32] and 5-LO 4C [21] were expressed in *Escherichia coli* BL21 (DE3) cells. Protein expression was started by addition of 0.2 mM IPTG (isopropyl-thio-β-D-galactopyranoside) after 5 h at 37 °C. Cells were harvested after 18 h at 22 °C. Purification of the enzymes was performed using ATP-affinity chromatography [33] and anion exchange chromatography on an ÄKTAxpress system (GE Healthcare, Uppsala, Sweden). In brief, after lysis, the supernatant of a 100,000 ×g centrifugation was loaded on an ATP-agarose column (column volume of 5 ml) and eluted with 50 mM phosphate/1 mM EDTA/20 mM ATP pH 7.4. The eluate was immediately loaded on a ResourceQ 1 ml column (GE Healthcare), washed with 50 mM phosphate/1 mM EDTA pH 7.4 and eluted with a linear gradient from 0% to 100% 50 mM phosphate/1 mM EDTA/0.5 M NaCl pH 7.4. The enzyme eluted at about 40%.

The concentration of 5-LO was determined using the Bio-Rad Bradford protein assay according to manufacturer's protocol, using bovine serum albumin as standard.

2.2.5. Measurement of 5-LO activity in intact cells

5×10^6 transfected HeLa cells or monocytes were resuspended in 1 ml of PBS with 1 mg/ml glucose and 1 mM calcium chloride. Cells were preincubated with or without 1 mM diamide at room temperature for 10 min. After addition of 5 μM A23187 and 3 μM AA, samples were incubated for 10 min at 37 °C. Reactions were stopped on ice by the addition of 1 ml methanol. 5-LO products were analyzed after solid phase extraction by HPLC as described before [23]. Data are shown as mean + standard error (SE) ($n \geq 3$).

2.2.6. Determination of 5-LO product formation of recombinant protein

0.5–10 μg of purified 5-LO protein were added to a reaction mixture of PBS containing 1 mM EDTA and 1 mM ATP with or without addition of 2 mM calcium chloride and/or 100 μg/ml PC. Samples were prewarmed for 30 sec at 37 °C and reaction was started by addition of 20 μM AA. Samples were stopped after 10 min at 37 °C on ice with 1 ml methanol. Resulting products were analyzed as described for intact cells. Data are shown as mean + SE ($n = 3$).

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