Biochimica et Biophysica Acta 1811 (2011) 1001–1010

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Tight association of N-terminal and catalytic subunits of rabbit 12/15-lipoxygenase is important for protein stability and catalytic activity

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ARTICLE INFO

Article history:
Received 25 June 2011
Received in revised form 9 August 2011
Accepted 15 August 2011
Available online 22 August 2011

Keywords:
Eicosanoids
Lipid peroxidation
Molecular enzymology
Structural biology
Motional flexibility
Thermal stability

ABSTRACT

12/15-Lipoxygenases (12/15-LOXs) have been implicated in inflammatory and hyperproliferative diseases but the structural biology of these enzymes is not well developed. Most LOXs constitute single polypeptide chain proteins that fold into a two-domain structure. In the crystal structure the two domains are tightly associated, but small angle X-ray scattering data and dynamic fluorescence studies suggested a high degree of structural flexibility involving movement of the N-terminal domain relative to catalytic subunit. When we inspected the interdomain interface we have found a limited number of side-chain contacts which are involved in interactions of these two structural subunits. One of such contact points involves tyrosine 98 of N-terminal domain. This aromatic amino acid is invariant in vertebrate LOXs regardless of overall sequence identity. To explore in more detail the role of aromatic interactions in interdomain association we have mutated Y98 to various residues and quantified the structural and functional consequences of these alterations. We have found that loss of an aromatic moiety at position 98 impaired the catalytic activity and membrane binding capacity of the mutant enzymes. Although CD and fluorescence emission spectra of wild-type and mutant enzyme species were indistinguishable, the mutation led to enlargement of the molecular shape of the enzyme as detected by analytic gel filtration and this structural alteration was shown to be associated with a loss of protein thermal stability. The possible role of tight interdomain association for the enzyme's structural performance is discussed.

1. Introduction

Lipoxygenases (LOXs) are lipid peroxidizing enzymes, which have been implicated in the pathogenesis of inflammatory [1,2] and hyperproliferative [3,4] diseases and thus, represent potential targets for pharmacological intervention [5,6]. All LOX-isoforms whose crystal structures have been determined so far indicate that these enzymes constitute single polypeptide chain proteins folded into a two-domain structure of the same topology [7–10]. The small N-terminal β-barrel domain resembles that of human lipases [11] and has been implicated in membrane binding [12]. Gene truncation of the N-terminal domain in rabbit 12/15-LOX reduces the catalytic efficiency of the enzyme, impaired membrane binding and accelerated suicidal inactivation [13,14]. In contrast, removal of N-terminal domain from soybean LOX1 (sLOX1) leads to a catalytic subunit exhibiting an improved catalytic efficiency [15] confirming the regulatory role of the N-terminal β-barrel domain. In all LOX crystal structures the two domains share a large interdomain interface and the two structural subunits are tightly interconnected by multiple non-covalent interactions. On the other hand, small angle X-ray scattering (SAXS) of the rabbit 12/15-LOX suggested that the N-terminal β-barrel domain might swing away from the catalytic subunit when in solution [16]. This conclusion was consistent with the high degree of motional flexibility of the enzyme as indicated by dynamic

Abbreviations: LOX(s), lipoxygenase(s); sLOX1, soybean lipoxygenase-1; LA, linoleic acid; 15-HETE, (15S,5Z,8Z,11Z,13E)-15-hydroxyeicosa-5,8,11,13-tetraenoic acid; 12-HETE, (12S,5Z,8Z,10E,14Z)-12-hydroxyeicosa-5,8,10,14-tetraenoic acid; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; IPTG, isopropyl-β-D-1-thio-galactopyranoside

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doi:10.1016/j.bbalip.2011.08.008

fluorescence measurements, fluorescence resonance energy transfer studies [17] and molecular dynamics simulations [18]. Moreover, re-evaluation of the X-ray coordinates of the rabbit 12/15-LOX inhibitor complex confirmed the structural flexibility of this isoenzyme since marked conformational alterations have been reported upon ligand binding at the active site [19]. These changes involve a 12 Å movement of a surface exposed α-helix and substantial rearrangement of active site constituents. When we inspected the interdomain interface of the rabbit 12/15-LOX for residues, which are involved in interaction between two structural subunits, we have noticed that Y98 of the N-terminal β-barrel domain significantly contributes to the interdomain contact plane (80 $Å^2$). Besides hydrophobic and van der Waals interactions the aromatic ring of Y98 may be involved in π–π interaction with Y614 of the catalytic domain. Homology study shows that Y at position 98 (rabbit 12/15-LOX nomenclature) is invariant in all mammalian LOX isoforms whereas Y614 is conserved only in 12/15-LOXs, but in other isoenzymes the residue at corresponding position is presented as aromatic H, thus, either π–π or cation–π interactions between structural subunits may represent a common feature of all mammalian LOX isoforms. In fact, recently performed SAXS measurements suggested that mutation of Y614 to positively charged R is not sufficient to induce a higher degree of interdomain movement. In contrast, simultaneous mutation of both Y614 and Y98 to R may force separation of the N-terminal domain from the rest of the molecule [20].

To explore the role of an aromatic side chain at position 98 of the rabbit 12/15-LOX for both stability and/or structural flexibility of the enzyme in more detail we first mutated Y98 to F, which retained the aromatic nature but removed the OH-group that might be involved in hydrogen bonding. Next, neutral (A) and positively charged (R) non-aromatic amino acids were introduced and the impact of these alterations on functional and structural enzyme properties was tested. Our results indicate that Y98 mutations to residues, which lack an aromatic system, altered the catalytic properties of the enzyme while the reaction specificity remained unchanged. This functional distortion has been related to structural changes, which are indicated by an increase in molecule diameter and is associated with a loss in thermal stability and higher oligomerization tendency.

2. Materials and methods

2.1. Materials

The chemicals used were obtained from the following sources: linoleic acid (9Z,12Z-octadecadienoic acid) from Serva (Heidelberg, Germany), HPLC standards of 12S-HETE, 15S-HETE from Cayman Chemicals, sodium borohydride, ampicillin from Life Technologies, Inc. (Eggenstein, Germany), and isopropyl-β-D-1-thiogalactopyranoside (IPTG) from Carl Roth GmbH (Karlsruhe, Germany). HPLC solvents were purchased from Baker (Deventer, The Netherlands). Restriction enzymes were obtained from Fermentas (St. Leon-Rot, Germany). Oligonucleotide synthesis was performed at BioTez (Berlin, Germany) and nucleic acid sequencing was carried out at Eurofins MWG Operon (Ebersberg, Germany).

2.2. Methods

2.2.1. Protein expression, purification and FPLC-analysis

Recombinant wild-type rabbit 12/15-LOX and its mutants were expressed in *E. coli* as His-tagged fusion proteins using a protocol that was modified according to reference [20]. Briefly, bacteria were transformed with the recombinant plasmids, and 18 l of LB medium containing 100 mg/l ampicillin was inoculated with a 30-ml overnight pre-culture per 1 l of medium. The bacteria were allowed to grow at 37 °C for 20 h, and then expression of the recombinant protein was induced by addition of 1 mM IPTG (final concentration). The cultures were kept for additional 3 h at 26 °C, the bacteria were pelleted, washed (PBS) and resuspended in 60 ml of PBS. Cells were disrupted using EmulsiFlex-C5 (Avestin, Canada) at 15 MPa and cell debris was spun down (30 min, 23,700×*g*). The clear lyses supernatant was applied to two 1.6-ml nickel–agarose columns (Qiagen). Each column was washed (4×2 ml) with buffer A containing 100 mM Tris–HCl, 300 mM NaCl, 10 mM imidazole (pH 8.0). The bound proteins were eluted with buffer B (100 mM Tris–HCl, 300 mM NaCl, 200 mM imidazole, pH 8.0). Five 0.6-ml fractions were collected, and the LOX activity was assayed. The fractions containing active protein were pooled and desalted using Ecno-PackR 10 DG columns (Bio-Rad, USA) for further purification using ÄKTA FPLC system (P-920 Pump coupled with UPC-900 monitor, GE Healthcare, Sweden). Anion exchange chromatography was performed on a ResourceQ 6-ml column (GE Healthcare, Sweden). The desalted proteins (8 ml) were loaded to the column and eluted with a linear gradient of buffers A and B. Buffer A: 20 mM Tris–HCl (pH 6.8 or 8.0), buffer B: 20 mM Tris, 1 M NaCl, same pH. Fractions of 1 ml were collected and those containing LOX protein were pooled. Aliquots of this preparation were then applied for gel filtration on a SuperdexTM 200 10/300 GL column (GE Healthcare, Sweden). The column was eluted with 20 mM Tris buffer containing 200 mM NaCl at pH 6.8 or 8.0. The final LOX preparations were concentrated to 0.5 to 3 mg/ml using Spin-XR 6 30 k MWCO concentrators (Corning, UK).

2.2.2. CD and fluorescence spectroscopy measurements

Protein unfolding was monitored by CD measurements at 220 nm using a Jasco-710 spectropolarimeter at different temperatures and a 0.1 cm quartz cuvette. The alpha helix and beta structure content was estimated with the Jasco software package. Dynamic fluorescence measurements were performed on a K2 spectrofluorometer (ISS, Champaign, IL) equipped with Glan–Thompson polarizer, using the phase-shift and demodulation technique. During all measurements a constant temperature in the sample holder was maintained by an external bath circulator and carefully checked by a thermocouple. A laser diode with emission wavelength at 300 nm was used as light source to monitor tryptophan fluorescence. Fluorescence emission was monitored through cutoff filter (WG 320) to avoid scattered light.

2.2.3. Dynamic light scattering

Light scattering measurements were performed on a Horiba (Kyoto, Japan) LB-500 dynamic light scattering nanoparticle size analyzer, equipped with a 650 nm, 5 mW laser diode. Data analysis was performed using the accompanying software based on a Fourier-transform deconvolution procedure.

2.2.4. Membrane binding assay

Membrane binding assays were carried out as described previously [13]. Briefly, 200 μg of sub-mitochondrial particles were incubated at room temperature with 3 μg of recombinant enzymes in 50 mM HEPES buffer containing 150 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT, pH 7.4 (total assay volume 25 μl) for 5 min. The samples were underlaid with 100 μl of a sucrose buffer (500 mM sucrose in the same buffer) and centrifuged for 15 min at 100,000×*g* at 4 °C. The supernatant was carefully removed and transferred to a separate tube, and albumin was added to reach a final concentration of 0.3 mg/ml. The supernatant proteins were then precipitated with trichloroacetic acid and the precipitate was centrifuged for 20 min at 20,000×*g*. The supernatant of the centrifugation step was discarded and the two pellets were reconstituted in 5 μl of 4-fold concentrated electrophoresis loading buffer (Roti-Load, Roth, Germany). After addition of 15 μl of water the samples were heated to 95 °C for 5 min and then applied to SDS-PAGE. After electrophoresis, the proteins were blotted to a nitrocellulose membrane and the blots were probed with a mouse anti-RGS-His tag antibody (Qiagen, Hilden, Germany). As secondary antibody a peroxidase-conjugated anti-mouse IgG antibody (Sigma, Deisenhofen, Germany)