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Role of Arg403 for thermostability and catalytic activity of rabbit 12/15-lipoxygenase

Almerinda Di Venere [a,b], Thomas Horn [c], Sabine Stehling [c], Giampiero Mei [a,b], Laura Masgrau [d], Àngels González-Lafont [d,e], Hartmut Kühn [c], Igor Ivanov [f,*]

[a] Department of Experimental Medicine and Surgery, University of Tor Vergata, Via Montpellier 1, 00133 Rome, Italy
[b] IRCCS Neuromed, Pozzilli, Italy
[c] Institute of Biochemistry, Charité-Universitätsmedizin Berlin, Chariteplatz 1, D-10117 Berlin, Germany
[d] Institut de Biotecnologia i de Biomedicina (IBB), Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain
[e] Departament de Química, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain
[f] Institute of Toxicology and Pharmacology, Universitätsmedizin Rostock, Schilling Allee 70, D-18059 Rostock, Germany

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ABSTRACT

12/15-Lipoxygenases (12/15-LOX) have been implicated in inflammatory and hyperproliferative diseases but the numerous aspects of structural biology of these enzymes are far from clear. Early mutagenesis data and structural modeling of enzyme–substrate complexes suggested that Arg403, which is localized at the entrance of the putative substrate binding pocket, might interact with the fatty acid carboxylic group. On the other hand, side-chain of Arg403 is a part of an ionic network with the residues of α2-helix, which undergoes pronounced conformation changes upon inhibitor binding. To explore the role of Arg403 for catalysis in more detail we exchanged positively charged Arg403 to neutral Leu and quantified structural and functional consequences of the alteration at the site of mutation using fluorometric techniques. We found that a loss of electrostatic interaction between Arg403 and negatively charged amino acid residues of α2-helix has only minor impact on protein folding, but partially destabilized the tertiary structure of the enzyme. We hypothesize that interaction of Arg403 with the substrate's carboxylate might be involved in a complex mechanism triggering conformational changes of the α2-helix, which are required for formation of the catalytically competent dimer r12/15-LOX complex at pre-catalytic stages.

1. Introduction

Lipoxygenases are iron-containing lipid peroxidizing enzymes that catalyze dioxygenation of polyunsaturated fatty acids to their corresponding hydroperoxy derivatives [1,2]. LOX genes have been detected in eukaryotes [3,4] and bacteria [5] but for the time being there is no report on LOX sequences in archaea. Mammalian lipoxygenases (LOXs) have been implicated in the pathogenesis of inflammatory and hyperproliferative diseases [6,7] but the detailed mechanism of action for the different LOX-isoforms is still a matter of discussion. Although the crystal structures of various LOX isoforms have been solved [8–12] there is no convincing X-ray data indicating the alignment of substrate fatty acids at the active site of wild-type enzyme species. Mutagenesis data and structural modeling of enzyme–substrate complexes suggested that for 12/15-LOX fatty acid substrate penetrates into the active site with its methyl terminus ahead, so that fatty acid carboxylic group might interact with Arg403 that is located at the entrance of the putative substrate binding pocket [13]. X-ray crystallography complex [10] showed that ligand binding (RS7-inhibitor) at the active site of rabbit 12/15-LOX (r12/15-LOX) induces conformational changes in the immediate surroundings of the catalytic center and α2- and α18-helices are particularly affected. In the inhibitor-bound form (monomer B) the external α2-helix has translocated away from its original position by about 12 Å to close the entrance into putative substrate-binding cavity, thus, making it inaccessible form the surface. As a part of this restructuring process initial residues of the α2-helix (167–174), which are located close to Arg403, become disordered and changes in the H-bond pattern of Arg403 are observed. In ligand-free molecule (monomer A), the positively charged Arg403 forms an ionic network with the side chains of Glu399 and Glu176 [10], whereas in the inhibitor-bound form this residue interacts mainly with the side chain of Asp174 and the carbonyl backbone of Ile173. The α18-helix containing Leu597 controls the shape and the size of the cavity. In the inhibitor-bound form it is displaced providing space for ligand. Substrate

Abbreviations: LOX(s), lipoxygenase(s); LA, linoleic acid; AA, arachidonic acid; Me-AA, methyl arachidonate; 15-HETE, (5Z,8Z,11Z,13E)-15-hydroxyeicosa-5,8,11,13-tetraenoic acid; 12-HETE, (5Z,8Z,10E,14Z)-12-hydroxyeicosa-5,8,10,14-tetraenoic acid; 5,15-DiHETE, (6E,8Z,11Z,13E)-5,15-dihydroxyeicosa-6,8,11,13-tetraenoic acid; 8,15-DiHETE, (5Z,9E,11Z,13E)-8,15-dihydroxyeicosa-5,9,11,13-tetraenoic acid; IPTG, isopropyl-β-D-1-thiogalactopyranoside

* Corresponding author. Tel.: +49 381 4945959; fax: +49 381 4945772.
E-mail address: igor_ivanov@gmx.de (I. Ivanov).

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(AA or LA) docking to liganded and unliganded conformations of the enzyme suggested that displacement of α18-helix is also required for correct substrate binding and catalysis.

In the past, LOXs have been believed to function as monomers. However, more recent investigations into the solution structure of rabbit 12/15-LOX suggested that this enzyme may form dimers in which two non-covalently linked enzyme molecules share joint interface involving residues of both α2- and α18-helices [14]. Modeling of the interface contacts between two r12/15-LOX monomers within a dimer favored the geometry of heterodimer reported in the crystal [10] and suggested that such complexes may be stable in the presence of arachidonic acid [14].

Molecular dynamics simulations of r12/15-LOX-arachidonic acid and of r12/15-LOX-linoleic acid complexes also suggest that upon substrate binding ionic interactions of Arg403 seen in the inhibitor-bound form might be disrupted [15,16]. In most simulations Arg403 maintains contact with the side chain of Glu176 (as seen in the ligand-free form) while interacting with the substrates' carboxylic group and with water molecules. However, in a number of simulations Arg403 does not form any contact to the residues of α2-helix but interacts only with the substrate carboxylate and water molecules. In those cases, α2-helix is observed to shift position. Thus, Arg403 might not only contribute to positioning the fatty acid carboxylate close to the entrance of the substrate binding cavity [13,17], but simultaneously, it might also participate in the conformational change of the α2-helix that accompanies ligand binding [15,16]. To obtain more direct evidence for this hypothesis we expressed Arg403Leu mutant of the r12/15-LOX in *Escherichia coli*, purified the recombinant protein from a bacterial lysate as described previously for the wild-type [14,18] and compared its structural and functional parameters with those of the wild-type enzyme.

2. Materials and methods

2.1. Materials

The chemicals used were obtained from the following sources: arachidonic acid (5Z,8Z,11Z,14Z-eicosatetraenoic acid) from Serva (Heidelberg, Germany); HPLC standards of 5(S)-HETE, 12(S)-HETE, 12(±)-HETE, 15(S)-HETE and 15(±)-HETE from Cayman Chemicals; sodium borohydride, ampicillin from Life Technologies Inc. (Eggenstein, Germany); isopropyl-β-D-thiogalactopyranoside (IPTG) from Carl Roth GmbH (Karlsruhe, Germany); and HPLC solvents from Baker (Deventer, The Netherlands). Restriction enzymes were purchased from New England Biolabs (Schwalbach, Germany). Oligonucleotide synthesis was performed at BioTez (Berlin, Germany) and nucleic acid sequencing was carried out at Eurofins MWG Operon (Ebersberg, Germany). The *E. coli* strain XL-1 blue was purchased from Stratagene (La Jolla, CA).

2.2. Methods

2.2.1. Site-directed mutagenesis and bacterial expression

The wild-type LOX and its mutant were expressed as N-terminal His-tag fusion proteins in *E. coli* as described before [18]. For this purpose the cDNAs were cloned into the pQE-9 prokaryotic expression plasmid in such a way that the starting methionine of the LOX coding sequence was deleted. Because of technical reasons the N-terminus was elongated by additional amino acids including six consecutive His. For the deletion of the N-terminal β-barrel domain a Sal I restriction site was introduced in front of Cys115 by PCR as described previously [19]. The PCR fragment was digested with Sal I and Kpn I, and ligated into the pQE-9 expression plasmid containing the wild-type 15-LOX and *E. coli* was transformed with this plasmid. This procedure led to a N-terminal truncation mutant that lacked the first 114 amino acids. Site-directed mutagenesis was performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands). For the large culture scale the 100 ml pre-culture was added to 18 l LB medium containing ampicillin (0.1 mg/l) and bacteria were grown at 37 °C over night. Expression of recombinant LOX was initiated by the addition of 1 mM IPTG followed by incubation of the culture for 3 h at 30 °C.

2.2.2. Enzyme purification

Wild-type enzyme, Arg403Leu mutant and C-terminal domain were purified to apparent electrophoretic homogeneity by sequential affinity chromatography on a Ni-NTA column, anion exchange and size-exclusion chromatography according to the protocol [20]. Briefly, the cells were spun down, re-suspended in PBS and lysed with an EmulsiFlex-C5 high pressure homogenizer (Avestin, Ottawa, Canada). The cell debris was spun down; the supernatant was added to 1.5 ml of Ni-NTA (Macherey-Nagel, Düren, Germany). The suspension was incubated for 1 h at 4 °C and centrifuged for 10 min (1700 ×*g*), and the precipitate was transferred to an open bed column. The column was washed with 50 mM NaH_2PO_4 buffer containing 300 mM NaCl (pH 8.0) and adhering proteins were eluted with 50 mM NaH_2PO_4 buffer containing 300 mM NaCl, and 200 mM imidazole (pH 8.0). The LOX activity of the elution fractions was tested employing the spectrophotometric assay. The pooled LOX fractions were desalted on an Econo-Pac 10DG column (Bio-Rad, Munich, Germany) and further purified by FPLC on a Resource Q column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) followed by the size-exclusion chromatography.

2.2.3. CD and fluorescence spectroscopy measurements

Protein unfolding was monitored by CD measurements at 220 nm using a Jasco-710 spectropolarimeter at different temperatures using a 0.1 cm quartz cuvette. Steady-state fluorescence spectra were recorded with a ISS-K2 fluorometer (ISS, Champaign, IL, USA) upon excitation at 280 nm. 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 cut-off filters (WG 320) to avoid scattered light. High pressure steady-state experiments were performed using the ISS pressure cell as described by Paladini and Weber [21]. The samples were thermostated at 20 °C using an external bath circulator. The pressure range was limited to 2500 bar due to the intrinsic resistance of the 1 cm quartz windows.

2.2.4. 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. A 1 μM enzyme solution in 20 mM Tris (pH 8.0) was filtered using GHP Acrodisc 13, 0.2 μM (Pall Gelman Laboratory, USA) filters and assayed without ligand. Then different concentrations of 13(S)-HODE were added (concentration range from 10 to 30 μM), the samples were kept on ice for 10 min and filtered again and the light scattering was performed.

2.2.5. GdnHCl denaturation studies

A stock solution of GdnHCl (8 M) was prepared in 20 mM Tris buffer (pH 8.0) and its aliquots were used to prepare a series of dilutions containing different concentrations of GdnHCl. The CD and fluorescence unfolding by GdnHCl was achieved by incubating the protein in the presence of different amounts of denaturant for 12 h at 4 °C. Each measurement was repeated at least three times, and the relative standard deviation was reported in the figures as an error bar. The reversibility of the process was checked obtaining protein refolding by diluting a highly concentrated, fully unfolded sample in