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Molecular basis for the catalytic inactivity of a naturally occurring near-null variant of human ALOX15

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ABSTRACT

Mammalian lipoxygenases belong to a family of lipid-peroxidizing enzymes, which have been implicated in cardiovascular, hyperproliferative and neurodegenerative diseases. Here we report that a naturally occurring mutation in the hALOX15 gene leads to expression of a catalytically near-null enzyme variant (hGly422Glu). The inactivity may be related to severe misfolding of the enzyme protein, which was concluded from CD-spectra as well as from thermal and chemical stability assays. *In silico* mutagenesis experiments suggest that most mutations at hGly422 have the potential to induce sterical clash, which might be considered a reason for protein misfolding. hGly422 is conserved among ALOX5, ALOX12 and ALOX15 isoforms and corresponding hALOX12 and hALOX5 mutants also exhibited a reduced catalytic activity. Interestingly, in the hALOX5 Gly429Glu mutants the reaction specificity of arachidonic acid oxygenation was shifted from 5S- to 8S- and 12R-H(p)ETE formation. Taken together, our data indicate that the conserved glycine is of functional importance for these enzyme variants and most mutants at this position lose catalytic activity.

1. Introduction

Lipoxygenases (LOX) are non-heme iron containing enzymes that catalyze the dioxygenation of poly-unsaturated fatty acids containing a *cis*/*cis* 1,4-pentadiene structure to their corresponding hydroperoxides [1,2]. LOX genes occur in a few bacteria, fungi, plants and animals but are lacking in archae [3–6]. According to the positional specificity of arachidonic acid oxygenation mammalian LOXs can be classified as 5-LOX, 8-LOX, 12-LOX and 15-LOX [1,2]. The 3D-structures of various mammalian LOXs have been solved [7–10] and despite subtle structural differences the published X-ray coordinates indicate a high degree of structural similarity between the different isoforms.

Human arachidonate lipoxygenases (hALOX) have been implicated in the pathogenesis of cardio-vascular [11–14] and neurodegenerative diseases [15] but the mechanistic basis for their patho-physiological role is controversially discussed. In fact, pro- [16–19] and anti-atherogenic [20–23] activities have been reported for hALOX15 in different animal atherosclerosis models and thus, the precise role of the human enzyme in atherogenesis remains to be clarified. In an attempt to shed a light on this question, different case–control studies have been carried out in which single nucleotide polymorphisms (SNPs) in the ALOX15 gene were correlated with different read out parameters of cardio-vascular diseases such as the frequency of ischemic stroke [24], coronary artery disease [25,26] and myocardial infarction [27]. Unfortunately, the functional consequences of these mutations, especially for those localized in the 3′-UTR (rs916055) and intronic gene regions (rs7217186 + rs2619112) have not been explored in detail. Functional studies for the non-synonymous hALOX15 SNP Thr560Met (rs34210653) in exon 13 have demonstrated that this mutation may cause partial destruction of the hydrogen bonding network connecting hThr560 with active site residues [28] and these structural alterations

Abbreviations: LOXs, lipoxygenases; ALOX, arachidonate lipoxygenase; 15-H(p)ETE, (5Z,8Z,11Z,13E)-15-hydroperoxyeicosa-5,8,11,13-tetraenoic acid; 13-H(p)ODE, (9Z,11E,13S)-13-hydroperoxyoctadeca-9,11-dienoic acid; 12-H(p)ETE, (5Z,8Z,10E,14Z)-12-hydroperoxyeicosa-5,8,10,14-tetraenoic acid; 8-H(p)ETE, (5Z,9E,11Z,14Z)-8-hydroperoxyicosa-5,9,11,14-tetraenoic acid; IPTG, Isopropyl-β-D-thiogalactopyranoside; HETE, hydroxyeicosatetraenoic acid; HpETE, hydroperoxyeicosatetraenoic acid; UTR, untranslated region

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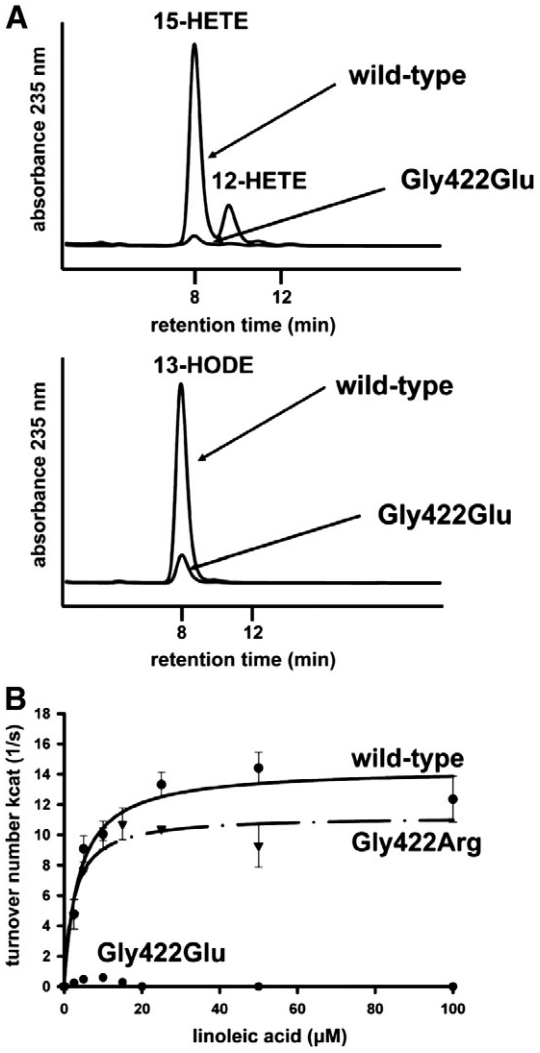

Fig. 1. Protein chemical end enzymatic characterization of human ALOX15 variants. (*A*) *Reverse-phase HPLC chromatogram of hALOX15 wild-type and hGly422Glu.* The RP-HPLC chromatogram shows that the wild-type has a major 15- and a minor 12-arachidonate oxygenation specificity and an exclusive 13-linoleate oxygenation specificity, which is typical for this LOX isoform; the hGly422Glu variant is nearly inactive and has an atypical reaction spectra. (*B*) *Michaelis–Menten kinetics of hALOX15 and hGly422Glu + hGly422Arg mutant.* The kinetic measurements with linoleic acid (LA) as a substrate show that the wild-type enzyme and the hGly422Arg mutant follow Michaelis–Menten and are therefore fitted with a hyperbolic equation whereas the hGly422Glu mutant is a nearly inactive enzyme variant.

seem to induce partial misfolding of the enzyme (see Supporting information S1).

Because of the potential role of the ALOX15 gene for atherogenesis, we searched the NCBI dbSNP databases for other non-synonymous nucleotide exchanges in the ALOX15 gene and found two rare mutations in the triplet coding for hGly422. These nucleotide exchanges lead to two non-conservative amino acid mutations (hGly422Glu, hGly422Arg). In the 3D-structure of rabbit ALOX15, which shares a high degree of amino acid conservation with the human ortholog enzyme, rGly423 (hG422) is located in the surrounding of rIle418 and rMet419, which have previously been described as position specificity determinants for mammalian ALOX15 isoforms [29–31]. Because of this structural proximity severe functional consequences have been expected for the hGly422Glu (rs61099320) and the hGly422Arg (rs147238486) exchange.

To test this hypothesis we expressed wild-type human ALOX15 and the corresponding enzyme mutants in *Escherichia coli* and characterized the purified enzymes. We found that the hGly422Glu exchange leads to a loss of catalytic activity, and more detailed structural data (CD, fluorescence measurements) show that the mutation seems to cause a change in the secondary structure composition of the protein. In contrast, the hGly422Arg mutant retained its catalytic activity and its structure is minorly affected.

2. Material and methods

2.1. Materials

The chemicals were obtained from the following sources: arachidonic acid (5Z,8Z,11Z,14Z-eicosatetraenoic acid), linoleic acid (9Z,12Z-octadeca-9,12-dienoic acid) and chloramphenicol from Sigma Aldrich (Hamburg, Germany), HPLC standards of 5(±)-HETE, 8(±)-HETE, 11(±)-HETE, 12(±)-HETE, 13(±)-HODE, and 15(±)-HETE from Cayman Chem. (distributed by Spi Bio, Montigny le Bretonneux, France), sodium borohydride, ampicillin from Life Technologies, Inc. (Eggenstein, Germany), kanamycin and isopropyl-β-thiogalactopyranoside (IPTG) from Carl Roth GmbH (Karlsruhe, Germany). HPLC solvents were ordered from Baker (Deventer, Netherlands) or VWR International GmbH (Darmstadt, Germany). Restriction enzymes were purchased from Fermentas (St. Leon-Rot, Germany). Oligonucleotide synthesis was performed at BioTez (Berlin, Germany) and DNA sequencing was carried out at Eurofins MWG Operon (Ebersberg, Germany). The *E. coli* strain XL-1 blue was purchased from Stratagene (La Jolla, CA), the *E. coli* strain BL21(DE3)pLysS and the fluorescent dye SYPRO Orange® were purchased from Invitrogen (Carlsbad, California, USA). The EnBase fed batch system was obtained from Biosilta (Berlin, Germany).

2.2. Methods

2.2.1. Recombinant expression and purification of hALOX15, hALOX5, hALOX12 and rabbit ALOX15

In order to express hALOX15 as a His-tag fusion protein, the coding region of the corresponding cDNA was first amplified by RT-PCR and then cloned into the pET28b expression vector between the *SalI* (N-terminus) and *HindIII* (C-terminus) restriction sites. For each mutant preparation a 0.5 l fed batch liquid culture (EnBase, Biosilta)

Table 1
Kinetic characterization of naturally occurring hALOX15 variants.
Purified recombinant hALOX15 wild-type and the natural occurring hGly422 mutants were incubated with arachidonic and linoleic acid (n = 4). The amounts for 15-HETE formation were quantified for each sample, and wild-type 15-HETE formation was set to 100%. The k_{cat} and K_m values of the hALOX15 variants for linoleic acid were taken from the hyperbolic equations of the Michaelis–Menten kinetics. The hGly422Glu mutant does not follow the Michaelis–Menten principle and therefore a determination of kinetic parameters with a hyperbolic equation is not possible.

hALOX15	Share of 15-HETE %	Rel. activity (AA) %	k_{cat}(LA) s^{-1}	K_m(LA) μM	R^2
Wild-type	83 ± 4	100 ± 1	14.4 ± 0.8	3.8 ± 1.0	0.89
Gly422Glu	83 ± 4	<1	n.d	n.d	n.d.
Gly422Arg	89 ± 7	36 ± 3	9.6 ± 0.7	1.7 ± 0.8	0.96