Redox Biology 1 (2013) 566–577

Contents lists available at ScienceDirect

Redox Biology

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

Functional characterization of genetic enzyme variations in human lipoxygenases

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ARTICLE INFO

Article history:
Received 28 October 2013
Accepted 1 November 2013

Keywords:
Eicosanoids
Leukotrienes
Lipoxygenases
Gene polymorphism
SNP

ABSTRACT

Mammalian lipoxygenases play a role in normal cell development and differentiation but they have also been implicated in the pathogenesis of cardiovascular, hyperproliferative and neurodegenerative diseases. As lipid peroxidizing enzymes they are involved in the regulation of cellular redox homeostasis since they produce lipid hydroperoxides, which serve as an efficient source for free radicals. There are various epidemiological correlation studies relating naturally occurring variations in the six human lipoxygenase genes (SNPs or rare mutations) to the frequency for various diseases in these individuals, but for most of the described variations no functional data are available. Employing a combined bioinformatical and enzymological strategy, which included structural modeling and experimental site-directed mutagenesis, we systematically explored the structural and functional consequences of non-synonymous genetic variations in four different human lipoxygenase genes (ALOX5, ALOX12, ALOX15, and ALOX15B) that have been identified in the human 1000 genome project. Due to a lack of a functional expression system we resigned to analyze the functionality of genetic variations in the hALOX12B and hALOXE3 gene. We found that most of the frequent non-synonymous coding SNPs are located at the enzyme surface and hardly alter the enzyme functionality. In contrast, genetic variations which affect functional important amino acid residues or lead to truncated enzyme variations (nonsense mutations) are usually rare with a global allele frequency $< 0.1\%$. This data suggest that there appears to be an evolutionary pressure on the coding regions of the lipoxygenase genes preventing the accumulation of loss-of-function variations in the human population.

Introduction

Lipoxygenases (LOXs) are lipid-peroxidizing enzymes that catalyze the dioxygenation of poly-unsaturated fatty acids containing a *cis,cis*-1,4-pentadiene structure [1]. Together with various oxygen activating enzymes LOXs increase the cellular oxidizing potential by biosynthesizing lipid hydroperoxides, which serve as an efficient source for free radicals unless they are reduced by peroxide reducing enzymes to the corresponding alcohols [2,3]. LOXs are widely distributed in terrestrial life since they occur in bacteria, fungi, lower marine organisms, plants and higher animals [4,5]. In humans six functional LOX genes (ALOX5, ALOX12, ALOX15, ALOX15B, ALOX12B, and ALOXE3) have been identified [1]. The crystal structures of three different mammalian LOX isoforms (human ALOX5, rabbit ALOX15, porcine ALOX15) have been solved [6–8] and despite subtle structural differences there is a high degree of structural similarity between different LOX isoforms. Human LOXs (hALOX) have been implicated in the pathogenesis of cardiovascular and inflammatory diseases as well as in various forms of cancer but the detailed mechanistic basis for their pathophysiological roles is still a matter of discussion [9–12].

In different epidemiological correlation studies genetic variations in hALOX genes have been related to an increased risk of colorectal cancer [13,14], stroke [15], bronchial asthma [16] and

Abbreviations: LOXs, lipoxygenases; ALOX, arachidonate lipoxygenase; 5-H(p)ETE, (6E,8Z,11Z,14Z)-5-hydroperoxyeicosa-6,8,11,14-tetraenoic acid; 15-H(p)ETE, (5Z,8Z,11Z,13E)-15-hydroperoxyeicosa-5,8,11,13-tetraenoic 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-hydroperoxyeicosa-5,9,11,14-tetraenoic acid; IPTG, Isopropyl-β-D-thiogalactopyranosid; HETE, hydroxyeicosatetraenoic acid; H(p)ETE, hydroperoxyeicosatetraenoic acid; LTA_4, 4-[(2S,3S)-3-[(1E,3E,5Z,8Z)-tetradeca-1,3,5,8-tetraen-1-yl]oxiran-2-yl]butanoic acid; LTB_4, 5(S),12(R)-dihydroxy-6,8,10,14-(Z,E,E,Z)-eicosatetraenoic acid; LTC_4, (5S,6R,7E,9E,11Z,14Z)-6-{[(2R)-2-[(4S)-4-amino-4-carboxybutanamido]-2-[(carboxymethyl) carbamoyl]ethyl]sulfanyl}-5-hydroxyeicosa-7,9,11,14-tetraenoic acid; UTR, untranslated region

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2213-2317
http://dx.doi.org/10.1016/j.redox.2013.11.001

atherosclerosis [17]. Unfortunately, most of these variations occur either in intronic, in the promoter or in other non-coding DNA regions and thus, functional characterization of these genetic variations is difficult. However, there are scattered reports, in which the functionality of some genetic variations has been studied: (i) A non-synonymous SNP in the hALOX12 gene (rs28395860) has been identified, which leads to a non-conservative Gln261Arg exchange. This mutation has been correlated to the pathogenesis of osteoporosis, breast cancer and schizophrenia [18–20]. Unfortunately, functional data on the recombinant enzyme indicated that this mutation did hardly impact the *in vitro* enzyme function [21]. (ii) Heterozygous allele carriers of the hALOX15 Thr560Met SNP (rs34210653) have a significant higher risk for coronary artery disease (CAD) [22]. *In vitro* mutagenesis studies indicated that this amino acid exchange leads to a near null variant of the enzyme and the molecular basis for the loss in the enzyme activity has been explored in detail [23]. A similar naturally occurring near null variant (rs61099320) of this enzyme has recently been described but because of its low occurrence frequency no epidemiological correlation study has yet been carried out [24]. (iii) Inactive enzyme variations in hALOX12B and hALOXE3 have been associated with a rare skin dysfunction called autosomal recessive congenital ichthyosis (ARCI) [25]. (iv) In a recent report the non-synonymous SNP rs2228065, which leads to a Glu254Lys exchange in hALOX5, was significantly associated with a higher risk of bronchial asthma [26], whereas it was not associated with an increased risk of breast cancer [27]. Unfortunately, no functional data are currently available for this particular enzyme variant.

Completion of the human 1000 Genome project provided an integrated map of genetic variations in 1092 individuals from all over the world [28] and the corresponding database (www.1000genomes.org) also includes a large number of genetic variations in enzymes and receptors of the arachidonic acid cascade. Although this database does not sufficiently cover the genetic diversity in all parts of the world, particularly in Southeast Asia [29], it serves as a suitable source for detailed studies on the functionality of naturally occurring genetic variants of enzymes, hormones, receptors, and structural proteins. The aim of this work was to analyze the functional aspects of non-synonymous coding enzyme variations in four different hALOX genes to find new interesting candidates which have strong functional effects on position- and stereospecificity, catalytic activity, thermostability and structure. Of course it is impossible to analyze all genetic variations and therefore, we mainly focussed on common SNPs with a published frequency and variations which are either non-conservative amino acid exchanges or who affect important amino acid residues. The N-terminal domain of these enzymes has mainly regulatory functions and even N-terminally truncated enzyme variations showed catalytic activity *in vitro* and therefore we focussed on genetic variations in the catalytic domain of these LOX isoforms. For this study we took advantage of the 1000 Genome Project database and evaluated the functionality of different genetic variations in four hALOX genes employing a combined *in silico* and *in vitro* approach, which involves recombinant protein expression, site-directed mutagenesis and computer-based structural modeling. Due to a lack of a functional expression system the genetic variations in hALOX12B and hALOXE3 are discussed on a theoretical basis.

Material and methods

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 Biomol, Hamburg, Germany), sodium borohydride from Aldrich (Steinheim, Germany), 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 the company Biosilta (Oulu, Finland).

Methods

Recombinant expression and purification of hALOX15, hALOX5, hALOX12 and hALOX15B

In order to express hALOX15 as a His-tag fusion protein, the coding region of the corresponding cDNA was first amplified by RT-PCR from human blood and then cloned into the pET28b expression vector between the *SalI* (N-terminus) and *HindIII* (C-terminus) restriction sites. For the wild-type enzyme and for each mutant a 0.5 l fed batch liquid culture (EnBase, Biosilta) of *E. coli* BL12(DE3)pLysS was grown overnight at 30 °C until an OD_{600} of about 15 was reached. Expression of the recombinant protein was then induced by addition of 1 mM IPTG (final concentration) and the culture was kept for 24 h at 23 °C until a final OD_{600} of 25–30. Bacteria were centrifuged (3,600*g*, 4 °C, 10 min) and resuspended in 45 ml phosphate buffered saline (PBS). Cells were lysed by sonification, the cell debris was removed by centrifugation (23,000*g*, 4 °C, 45 min), and the lysis supernatant was loaded onto a 2 ml Ni-NTA agarose affinity column. To remove loosely bound proteins, the column was washed subsequently with 100 mM Tris/HCl, 300 mM NaCl (pH 8.0) containing either 10 or 25 mM imidazole. The *His*-tagged fusion protein was then eluted with 100 mM Tris/HCl, 300 mM NaCl, 200 mM imidazole (pH 8.0). The elution fractions were desalted and further purified by anion exchange chromatography on a ResourceQ column (6 ml) to electrophoretic homogeneity (purity > 95%). The purified enzyme was stored in 100 mM Tris/HCl, 150 mM sodium chloride in the presence of 10% (v/v) glycerol (pH 8.0) at −80 °C until use. The iron content of the final LOX preparation was measured by atom absorbance spectroscopy on a Perkin-Elmer Life Sciences AA800 instrument equipped with an AS800 auto sampler. The iron content was related to LOX protein that was quantified spectrophotometrically (1 mg/ml pure human ALOX results in an absorbance of 1.6 at 280 nm).

Expression of hALOX12, hALOX15B and hALOX5 variants was carried out as previously described [30–31].

Lipoxygenase activity assays

ALOX activity of the purified LOX preparations was assayed by RP-HPLC quantification of arachidonic acid (AA) oxygenation. Purified enzyme (4 μg) was incubated with 100 μM arachidonic acid for 15 min at 37 °C. The hydroperoxide fatty acids were reduced to the corresponding alcohols using sodium borohydride and the sample was acidified with acetic acid to pH 3. Then 500 μl

of ice-cold methanol was added, protein precipitate was centrifuged (14,000*g*, 8 °C, 10 min) and aliquots of the clear supernatant were injected to RP-HPLC.

Kinetic measurements were performed using an UV-2102 UV/VIS Scanning Spectrophotometer (Shimadzu, Japan) measuring the increase of absorbance at 235 nm. When linoleic acid was used as a substrate 1.6 μg of purified enzyme was incubated with different concentrations of this fatty acid (1–100 μM) in 1 ml PBS and the formation of conjugated dienes was followed at 235 nm over 60 s.

Activity assays for hALOX12, hALOX15B and hALOX5 variants were carried out as previously described [30–31].

LTA_4 Synthase activity of hALOX5 was measured using a modified ALOX5 activity assay [31], while 25 μM 5 S–H(p)ETE was used as substrate. The reaction was stopped after 30 min by adding ice-cold methanol to the reaction mix in the ratio of 1.5:1. The total mix was then analyzed by RP-HPLC measuring the formation of conjugated trienes at 270 nm.

HPLC analysis

HPLC of the LOX products was performed on a Shimadzu LC-20 instrument equipped with an auto sampler recording the absorbance of the effluent at 235 nm. Reverse phase-HPLC was carried out on a Nucleodur C18 Gravity column (Macherey-Nagel, Düren, Germany; 250 × 4 mm, 5 μm particle size) coupled with a guard column (8 × 4 mm, 5-μm particle size). A solvent system of methanol/water/acetic acid (85/15/0.05, by vol) was used at a flow rate of 1 ml/min. Straight phase-HPLC (SP-HPLC) was performed on a Nucleosil 100-5 column (250 × 4 mm, 5 μm particle size) with the solvent system n-hexane/2-propanol/acetic acid (100/2/0.1, by vol) and a flow rate of 1 ml/min. Enantiomers of hydroxylated fatty acids were separated by chiral phase HPLC (CP-HPLC) employing different types of Chiralcel (Daicel Chem. Ind., Ltd.) columns. As mobile phase a mixture of n-hexane/2-propanol/acetic acid at a flow rate of 1 ml/min was used, in which the 2-propanol content was varied for the different positional isomers: 15-HETE: Chiralcel OD column, 5% 2-propanol; 5-HETE: Chiralcel OB column, 4% 2-propanol. 8- and 12-HETE were additionally separated by a solid-phase HPLC and then methylated using diazomethane. Enantiomers of 8-HETE-methylester were separated on a Chiralcel OB column with a solvent system n-hexane/2-propanol/acetic acid (100/4/0.1, by vol) and 12-HETE-methylester enantiomers were separated on a Chiralcel OD column with a solvent system (100/2/0.1, by vol).

Thermal shift assay (thermal denaturation)

Purified LOX enzyme (3 μM) was incubated with a 2.5-fold molar excess of fluorescent dye SyproOrange® in 20 mM Tris/HCl containing 150 mM NaCl (pH 8.0), total volume 20 μl. The heat denaturation reaction was performed on a Rotor-Gene RG-3000 real-time PCR machine (Corbett Research, Qiagen, Hilden, Germany) heating up the probes from 30 to 95 °C at a rate of 0.5 °C/s. The fluorescent dye SyproOrange® is quenched in aqueous solutions and becomes detectable after binding to hydrophobic molecules. Thermal unfolding of the protein induces surface exposure of hydrophobic core regions, where the dye is able to bind. Fluorescence intensity was quantified using a FAM/SYBR green filter. The software Rotor-Gene 4.6 was used for calculation of the negative first derivative of the raw data. Figures were generated with SigmaPlot. The melting temperature (T_m) for each mutant was analyzed in 3–4 independent experiments.

Table 1
Non-synonymous coding variations in six human lipoxygenase genes published in the 1000 Genome database (www.1000genomes.org).

ALOX isoform	sum	Number of non-synonymous coding variations		
		SNPs (Frequency > 1%)	Rare missense mutations (Frequency < 1%)	Nonsense mutations
ALOX15	94	2	84	8
ALOX5	69	1	68	0
ALOX12	67	2	64	1
ALOX15B	86	6	76	4
ALOX12B	69	1	68	0
ALOXe3	83	2	75	6
total	**468**	**14**	**435**	**19**

Immunoblotting

To quantify the relative amounts of different hALOX12 and hALOX5 variations in different enzyme preparations, immunoblotting was carried out as previously described using an anti-his-tag antibody [30–31].

Structural modeling, analysis and amino acid alignment

The crystal structure of the rabbit ALOX15 (PDB: 2P0M) was used as template to model the 3D-structure of the human enzyme. The rabbit ALOX15 shares a high degree of amino acid identity (> 80%) with the human ortholog and thus such modeling is plausible. The structure of the stable ALOX5 (PDB: 3O8Y) was used as for structural analysis of hALOX5 variations. A protein model from the Protein model database (PMDB) was used for structural analysis of human ALOX15B (PMDB code: PM0078035) [32]. The models for hALOX12 and hALOXE3 were generated with the homology modeling web server M4T (Albert Einstein College of Medicine, New York USA) [33–34]. The resulting structures were refined using the protein structure refinement server 3Drefine [35]. All structures were then solvated in TIP3 explicit water models and subjected to energy minimization in the program NAMD [36]. CHARMM27 [37] force field parameters were used and structures were minimized by 250,000 steps. Model quality estimation was performed with the Qmean quality estimation server [38]. The visualization software program PyMol (Schrodinger, New York, USA) was used for structural analysis and figure preparation. Multiple sequence alignment was performed with the ClustalW2 program (European Bioinformatics Institute, Cambridge, UK) setting default parameters. ALOX sequences were extracted from the NCBI database (Bethesda, USA) and the information about the natural occurring hALOX variations was taken from the 1000 Genome database (www.1000genomes.org).

Results

ALOX15 (human 15-lipoxygenase)

Human ALOX15 consists of 662 amino acids and its sequence was first published in 1988 [39]. In the 1000 Genome database 94 non-synonymous coding variations are described (Table 1). Two of them, Pro617Ser and Thr560Met, have a global allele frequency of > 1% and are therefore classified as SNPs (Table S1). Since it is impossible to test the functional consequences of all existing genetic variants we selected five non-conservative amino acid exchanges (Arg205Gln, Gly422Glu, Gly422Arg, Thr560Met, and Pro617Ser) in the catalytic domain of the enzyme for more detailed investigations. These mutants together with the wildtype enzyme were recombinantly expressed, purified to apparent

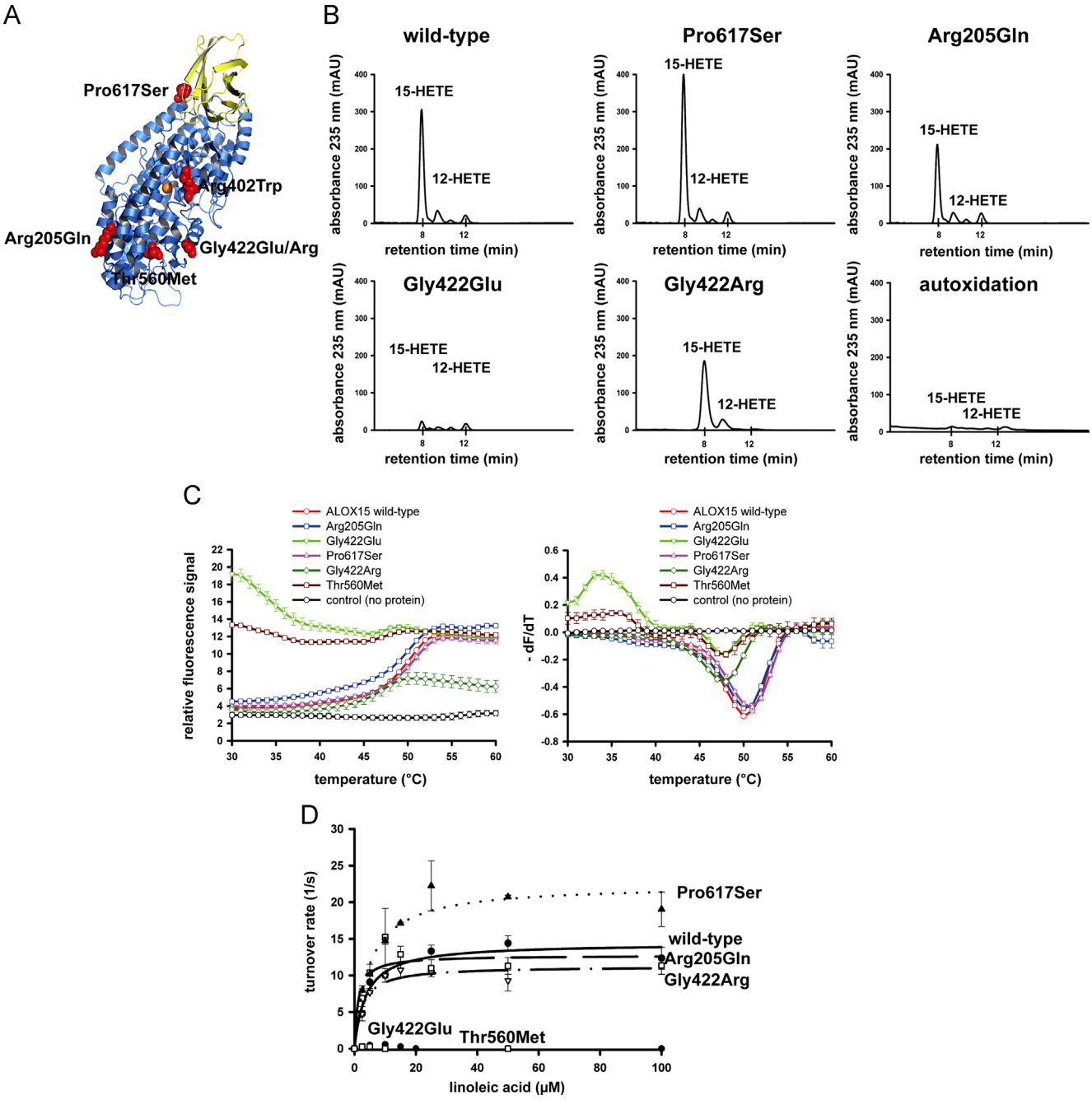

Fig. 1. Genetic variations in human ALOX15 (A) Structural localization of analyzed hALOX15 variations (PDB: 20PM). The N-terminal β-barrel domain is shown as yellow cartoon; the catalytic domain is represented as marine blue cartoon. The amino acid variations are shown as red spheres. (B) Reverse-phase HPLC of hALOX15 wildtype and different genetic variations. The chromatograms show that the wildtype and the Pro617Ser, Gly422Arg, Arg205Gln variations have a major 15- and minor 12- arachidonate oxygenation specificity while the Gly422Glu variation is inactive. The small peaks in the HPLC profile originate from arachidonic acid autoxidation. (C) Thermostability of hALOX15 wildtype and different genetic variations using a fluorescent-based thermodynamic shift assay. The thermal denaturation curve of the Pro617Ser, Arg205Gln, Gly422Arg variations are similar to the wildtype enzyme, whereas the Gly422Glu variation is different. The negative first derivative is calculated to determine the inflection point (T_m) of the unfolding curve more accurately. (D) Michaelis–Menten kinetics of different hALOX15 variations. The wildtype enzyme and the Pro617Ser, Arg205Gln, Gly422Arg variations follow Michaelis–Menten kinetics whereas the Thr560Met and Gly422Glu variations are catalytically silent.

homogeneity and characterized with respect to their enzymatic properties.

When we analyzed the structural position of the selected variations in the 3D-structure of the ALOX15 enzyme we found that two variations (Arg205Gln, Pro617Ser) (Fig. 1A) are localized on the enzyme surface with no immediate connection to the active site. In contrast, the Thr560Met and Gly422Glu/Arg are buried deeply inside the protein in close proximity to the active site (Fig. 1A). This localization might explain why these variants (Thr560Met, Gly422Glu) were catalytically silent (Table 2) which is consistent with previous reports [23–24]. The surface exposed mutants (Arg205Gln, Pro617Ser) exhibited a similar specific dioxygenase activity as the wildtype enzyme. Moreover, the reaction specificity was hardly altered since 15-H(p)ETE was the major (74–96%) arachidonic acid oxygenation product for both enzyme variants (Fig. 1B). To explore the structural consequences of the amino acid exchanges we monitored the thermostability of wild-type and mutant enzymes. We found that the enzymatic active

Table 2
Relative catalytic activities and melting points of different hALOX15 variations.

hALOX15	variation ID	Global allele frequency (%)	15-HETE (%)	Rel. activity (%)	Melting point T_m (°C)
wildtype			93 ± 4	100 ± 6	50.0 ± 0.6
Arg205Gln	rs11568101	0.5	89 ± 6	89 ± 7	50.4 ± 0.1
Arg402Trp	rs144038526	0.1	77 ± 2	36 ± 3	50.5 ± 0.2
Gly422Glu	rs61099320	< 0.1	83 ± 4	< 1	n.d.
Gly422Arg	rs147238486	0.1	87 ± 2	46 ± 4	47.6 ± 0.1
Thr560Met	rs34210653	1.5	74 ± 5	< 2	n.d.
Pro617Ser	rs41432647	1.0	96 ± 1	141 ± 17	51.0 ± 0.2

Purified recombinant hALOX15 wildtype and the different variations were incubated with arachidonic acid as described in Material and Methods ($n=4$). The amounts for 15-HETE were quantified for each sample, and wild-type 15-HETE formation was set to 100%. Thermal shift assays were performed as described in Material and Methods. The inflection point of the first negative derivative represents the melting point (T_m) of the enzyme variation.

Table 3
Kinetic parameters of different hALOX15 variations.

hALOX15	variation ID	k_{cat} (s^{-1})	K_m	R^2
wildtype	–	14.4 ± 0.8	3.8 ± 1.0	0.89
Arg205Gln	rs11568101	12.7 ± 1.2	1.2 ± 0.8	0.84
Gly422Glu	rs61099320	–	–	–
Gly422Arg	rs147238486	9.6 ± 0.7	1.7 ± 0.8	0.96
Thr560Met	rs34210653	–	–	–
Pro617Ser	rs140549513	22.4 ± 1.5	4.8 ± 1.3	0.95

Purified recombinant hALOX15 wildtype and the different variations were incubated with linoleic acid as described in Material and Methods ($n=3$). The formation of conjugated dienes was followed by recording the increase in absorbance at 235 nm for 60–120 s on the spectrophotometer. The resulting turnover rates (k_{cat}) and Michaelis constants (K_m) are given. The R square value (R^2) represents the accuracy of the hyperbolic equation.

variants showed a similar denaturation behavior as the wildtype enzyme, while the melting curves for the inactive enzyme variations (Gly422Glu, Thr560Met) were quite different (Fig. 1C). For these enzyme species the fluorescence levels were already high at lower temperatures (30 °C) and did not significantly increase when the temperature was elevated. In fact, for these enzyme species a true melting temperature (T_m) could not be determined (Fig. 1C). Mechanistically, this data suggest a high degree of misfolding in the corresponding enzyme variants, which is a plausible reason for their inactivity.

The kinetic properties of the catalytically active enzyme species (Arg205Gln; Pro617Ser; Gly422Arg) were not dramatically impaired by the genetic variations, since they had similar kinetic parameters (Fig. 1D) as the wildtype enzyme with low K_m values for linoleic acid (1.2–4.8 μM) and molecular turnover rates (k_{cat}) between 9.6 and 22.4 s^{-1} (Table 3).

For all LOX enzymes described so far the C-terminal amino acid functions as iron ligand and previous mutagenesis studies showed that deletion of this residue leads to a loss of catalytic activity [4]. In the 1000 Genome database eight nonsense mutations have been identified (Table 1). These genetic variations are likely to encode for truncated and inactive enzyme species and homozygous allele carriers should be completely hALOX15 deficient. Unfortunately, for neither of these nonsense mutations a global allele frequency has been reported suggesting that this mutation was only found once in the 1000 Genome database. In other words, the allele frequency should be < 0.1% (Table S1.).

Next we specifically searched in the 1000 Genome database for genetic variations of other functionally important amino acid residues (iron ligands, specificity determinants). All LOXs identified so far carry a redox active metal (iron or manganese) in the active site [1,4] and for hALOX15 His360, His365, His540, His544, and the C-terminal Ile662 constitute the direct iron ligands [6]. Neither of these residues was affected by a genetic variation.

hALOX15 exhibits a dual positional specificity [4] and the side chains of Phe352, Ile417, Ile592 play a major role for this enzyme property [40]. Analyzing the data obtained in the 1000 Genome database we found a rare variation at Phe352, in which the bulky Phe is exchanged to a less space-filling Leu (rs143365387). According to the triad concept, this enzyme variant should be a 12-lipoxygenating enzyme species and previous mutagenesis studies confirmed this conclusion [40]. This data indicate that homozygous allele carriers express a 12-lipoxygenating enzyme species and it would be of major interest whether such humans have any functional defects. Unfortunately, because of the low allele frequency the search for such homozygous allele carriers is rather laborious and the existence of such individuals is even questionable.

Previous amino acid alignments indicated that most S-LOXs carry an Ala at a critical position (Coffa/Brash determinant) whereas R-LOXs have a small Gly at this position. Although this concept is not applicable for all lipoxygenases [41] mutagenesis studies on different isoforms showed that Ala-to-Gly exchanges at this position increase the share of R-HETE isomers [41–42]. In hALOX15 Ala403 is the corresponding residue but so far there is no human genetic variation described for this position.

Membrane binding upregulates the catalytic activity of ALOX15 [43] but it is also a prerequisite for LOX catalyzed oxygenation of membrane lipids [43]. Surface exposed hydrophobic amino acids such as Tyr15, Leu70, Leu71, Lys180 and Leu194 have been implicated in membrane binding [44] and the human 1000 genome project has published genetic variations for two of these residues (Table S1.). Tyr15His (rs13306168) has a global frequency of 0.6% whereas no frequency data are available for the Leu194Pro exchange (rs11867874). Both mutations constitute more or less conserved amino acid exchanges and thus, only minor effects on membrane binding might be predicted.

In silico docking studies and molecular dynamic simulations with different fatty acid substrates suggested that Arg402 and Phe414 may be involved in substrate binding [45–46]. For Arg402 two rare genetic variations with a global frequency < 1% have been identified in the 1000 Genome database [Arg402Gln (TMP_ESP_17_4536752) and Arg402Trp (rs144038526)]. To obtain information of the functional relevance of these amino acid exchanges we expressed the Arg402Trp mutant of hALOX15 and performed activity and thermostability assays. Arg402Trp has a reduced catalytic activity (36% residual activity) while its thermostability and positional specificity was hardly affected. Nevertheless the reduced catalytic activity is not as dramatic as it is for two previous mentioned loss-of-function mutations, so one can assume that this variation has only minor functional effects (Table 2).

ALOX5 (human 5-lipoxygenase)

hALOX5 consists of 674 amino acids and its sequence was first published in 1988 [47]. It is the key enzyme in the biosynthesis of leukotrienes [1,48] and converts arachidonic acid (AA) mainly to 5S-H(p)ETE. In addition, it catalyzes the dehydration of 5S-H(p)ETE to the allylic epoxide leukotriene A4 (LTA_4) which can then be further metabolized by other enzymes (LTA4H, LTC4S, γ-glutamyl transferases, dipeptidases) to leukotrienes [1,48]. Leukotrienes (LTs) are pro-inflammatory mediators, which play a role in the pathogenesis of bronchial asthma and allergic rhinitis [1]. In the 1000 Genome database there are 69 non-synonymous coding variations described for hALOX5 (Table 1) but only the hALOX5 Glu254Lys (rs2228065) variant has a global frequency > 1% and is therefore defined as SNP. We selected seven non-synonymous coding variations in the catalytic domain of this enzyme for more

Table 4
Relative catalytic activities and product specificities of different hALOX5 variations.

hALOX5	variation ID	Global allele frequency (%)	5-HETE (%)	Rel. activity (%)
wildtype			74 ± 1	100 ± 2
Glu254Lys	rs2228065	7.5	90 ± 1	58 ± 5
Pro337Ser	rs28395860	0.1	85 ± 1	54 ± 1
Ala447Ser	rs28395869	0.0	81 ± 1	118 ± 5
Ala549Val	rs113388968	< 0.1	84 ± 1	22 ± 1
Pro577Leu	rs143439556	< 0.1	89 ± 1	95 ± 4
Thr591Met	rs146768497	< 0.1	86 ± 1	130 ± 7
Lys656Gln	rs147238486	0.0	80 ± 2	122 ± 3

hALOX5 aliquots of the Co-Sepharose elution fraction 2 containing equal amounts of LOX protein as determined by western blot analysis were used for activity assays ($n=4$). The amounts of HETE isomers were analyzed for each sample by HPLC and are used to calculate the catalytic activity of the different enzyme species. The HETE formation of the wildtype was set to 100%.

detailed functional analysis (Table 4). Structural localization of the altered amino acids revealed that except the Ala549Val variation, which is buried deeply inside the enzyme, all other variations are localized at the enzyme surface suggesting that they might have only minor effects on enzyme functionality (Fig. 2A). Site directed mutagenesis confirmed this hypothesis since the enzyme mutants exhibited similar reaction specificities and specific dioxygenase activities (Fig. 2B.). All mutants converted arachidonic acid to 5-H(p)ETE as the major reaction product (Fig. 2B) and their relative catalytic activities varied between 22% and130% when compared to the wildtype enzyme (Table 4). The Ala549Val variation (rs113388968) shows the strongest reduction of dioxygenase activity (loss of 78%) and this impairment might be related to its close proximity to the iron ligand sphere [7]. We also analyzed the effect of two genetic variations on the LTA_4 Synthase activity of hALOX5 and found that it was hardly affected by the two analyzed variations (Glu254Lys, Thr591Met) and this is consistent with our data for dioxygenase activity (Table S2.).

In contrast to ALOX15, no nonsense variation that leads to the expression of a C-terminally truncated enzyme has been described for hALOX5 in the database (Table 1).

As for ALOX15 we specifically searched the 1000 Genome database for genetic variations of other functionally important amino acid residues (iron ligands, specificity determinants, FY-cork, activator binding sites). His368, His373, His551, Asn555 and the C-terminal Ile674 coordinate the non-heme iron [7,48] but no variation has been described for any of these residues. Similarly, we did not find any mutants (Table S1) for the triad residues [49] and for the enantioselectivity determinant [41–42]. The substrate binding pocket of hALOX5 is an elongated cavity, which appears to be sealed at both ends for the ligand free protein. Phe178 and Tyr182 (FY-cork) close the cavity at one end [7] and Trp148 may block the opposite entrance to the cavity. Neither for the FY-cork, nor for Trp148 has any genetic variations been described.

There are numerous hALOX5-activating factors (Ca^{2+}, phosphatidylcholine, ATP, coactosin-like protein) and for some of them the binding mechanisms have been identified. For instance, Asn44, Asp45, and Glu47 are required for Ca^{2+}-binding [1,48]. For binding of the enzyme to biomembranes and phosphatidyl choline (PC) vesicles Trp13, Trp75, and Trp102 appear to be important [1,48]. However, for none of these residues genetic variations have been described.

In vitro kinase assays suggested that phosphorylation of specific amino acid residues (Ser271, Ser523, and Ser663) regulates the enzyme functionality [1,48]. There is a rare genetic variation at Ser271 in the database (Ser271Ile, TMP_ESP_10_45920561), which should prevent enzyme phosphorylation at this position.

ALOX12 (human platelet 12-lipoxygenase)

hALOX12 contains 663 amino acids and was the first mammalian lipoxygenase described back in 1974 [50]. In the 1000 Genome database there are 67 non-synonymous coding variations for this enzyme (Table 1). Two of them, Gln261Arg (rs28395860) and Asn322Ser (rs434473) have a high global allele frequency < 1% (38.8% and 34.4% respectively) (Table S1.). In total we analyzed four selected non-synonymous coding variations in the catalytic domain of the hALOX12 gene (Asp134His, Glu259Lys, Gln261Arg, and Asn322Ser). Structural analysis indicated that all of these amino acid exchanges are located on the enzyme surface and therefore only minor functional effects can be expected (Fig. 3A). In fact, the specific activity of all mutants was comparable (60-109%) to that of the wildtype enzyme (Fig. 3B) and 12-H(p)ETE was always the dominant reaction product for all enzyme variations (Table 5). Interestingly, the Gln261Arg exchange was also found in two *Homo Neanderthalensis* individuals [51].

One premature stop variation (Arg348stop, rs141346813) leading to a C-terminally truncated enzyme was found in the 1000 Genome database but since no frequency data are given it must be classified as rare enzyme mutation.

Similar to hALOX15 and hALOX5 we specifically searched the 1000 Genome database for genetic variations of other functionally important amino acid residues (iron ligands, position- and enantioselectivity determinants). The catalytic iron ion is coordinated by the residues His360, His365, His540, Asn544, and Ile663. The triad concept is partly applicable for this enzyme [52] and the hALOX12 carries an Ala at the critical enantioselectivity position 403 indicating that it is an S-LOX [41–42]. Neither for the iron coordination nor for the position- (Phe353, Ala417, and Ile593) and enantioselectivity determinants (Ala403) has any genetic variation been described (Table S1.). As for hALOX15 Arg402 of hALOX12 may play a role for positioning of the fatty acid substrate within the active site [45–46]. Arg402Gln (rs143539715) is a rare enzyme variant of this LOX isoform but the functional consequences of this amino acid exchange have not been explored.

ALOX15B (human 15-lipoxygenase type II)

This enzyme consists of 676 amino acids (Isoform D) and was first discovered in 1997 [53]. 86 non-synonymous coding variations have been identified in the 1000 Genome database and six of them have a global allele frequency > 1% (Table 1): Pro77Ala (rs78230493), Ala311Val (rs148602477), Arg486His (rs9895916), Arg635Gln (rs61730298), Gln656Arg (rs4792147), and Ile676Val (rs7225107) (Table S.1). We selected three frequent genetic variations (Arg486His, Gln656Arg and Ile676Val) in the catalytic domain of the enzyme for more detailed characterization. Two of them (Arg486His, Gl656Arg) are located at the enzyme surface and therefore only minor functional effects were expected. In contrast, Ile676 is an iron ligand and Ile676Val exchange might impact the functionality of the enzyme (Fig. 4A). Functional analysis showed that none of the analyzed hALOX15B SNPs dramatically altered the position specificity (Fig. 4B) and specific activity (Table 6) of the enzyme.

Thermal denaturation studies (Fig. 4C) indicated a two-state unfolding curve for all analyzed hALOX15B variations and analysis of the first derivative of the denaturation curves showed that the Ile676Val mutant has a slightly reduced melting temperature (T_m). This data suggest that the Ile676Val exchange destabilizes the enzyme structure to a certain degree. In contrast, the wildtype enzyme, the Arg486His and Gln656Arg mutants have similar melting curves and thus, these mutations did not impact the enzyme structure (Table 6). The basic kinetic constants K_m and k_{cat} (Fig. 4D) were similar for the wildtype enzyme and the different

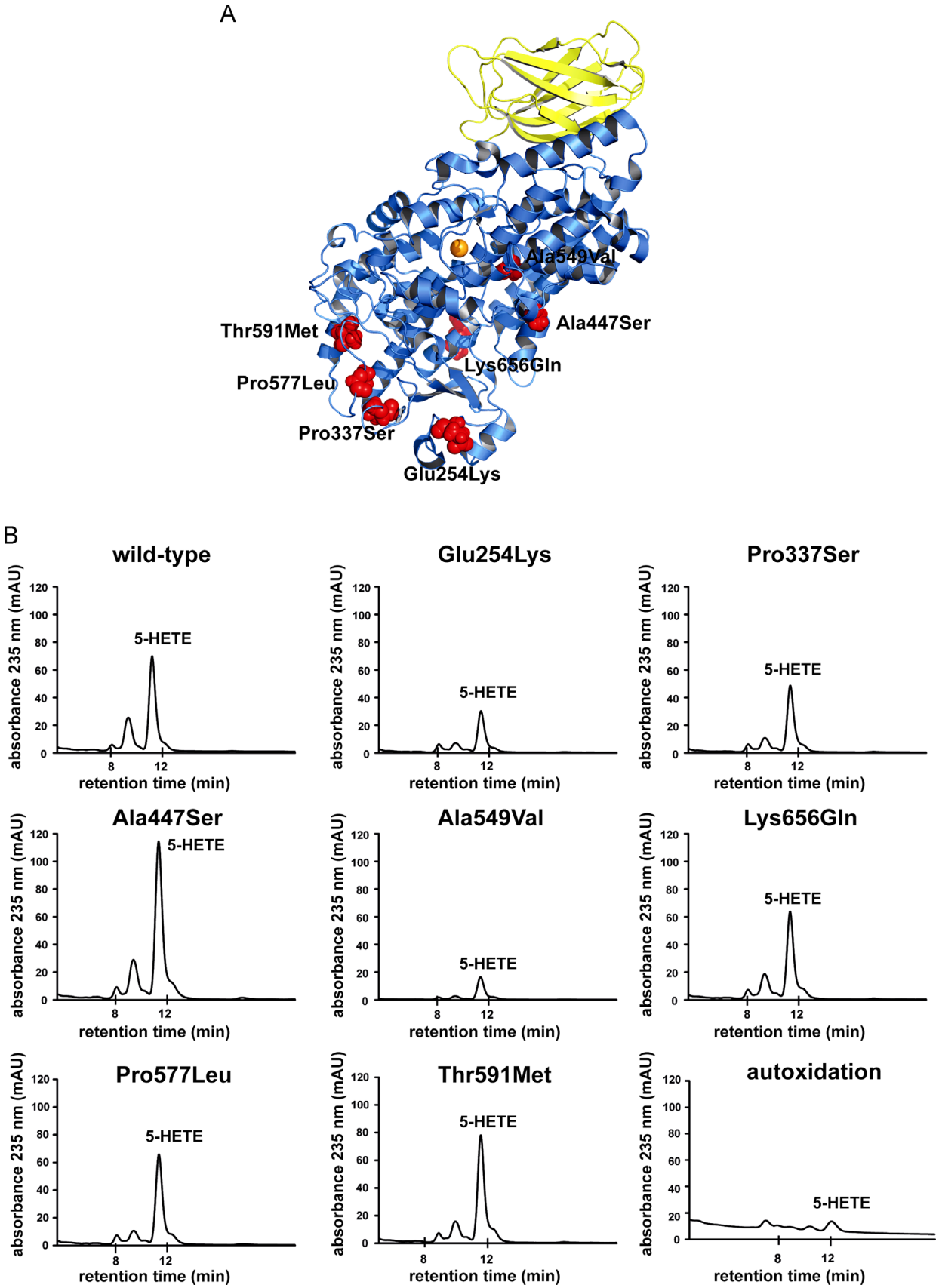

Fig. 2. Genetic variations in human ALOX5 (A) Structural localization of analyzed hALOX5 variations (PDB: 3O8Y). The N-terminal β-barrel domain is shown as yellow cartoon; the catalytic domain is represented as marine blue cartoon. The amino acid variations are shown as red spheres. (B) Reverse-phase HPLC chromatogram of hALOX5 wildtype and different genetic variations. The chromatograms show that the wildtype and the hALOX5 variations are major 5-lipoxygenating enzymes. The Ala549Val variation shows the strongest reduction in catalytic activity but also forms 5-H(p)ETE as major oxygenation product.

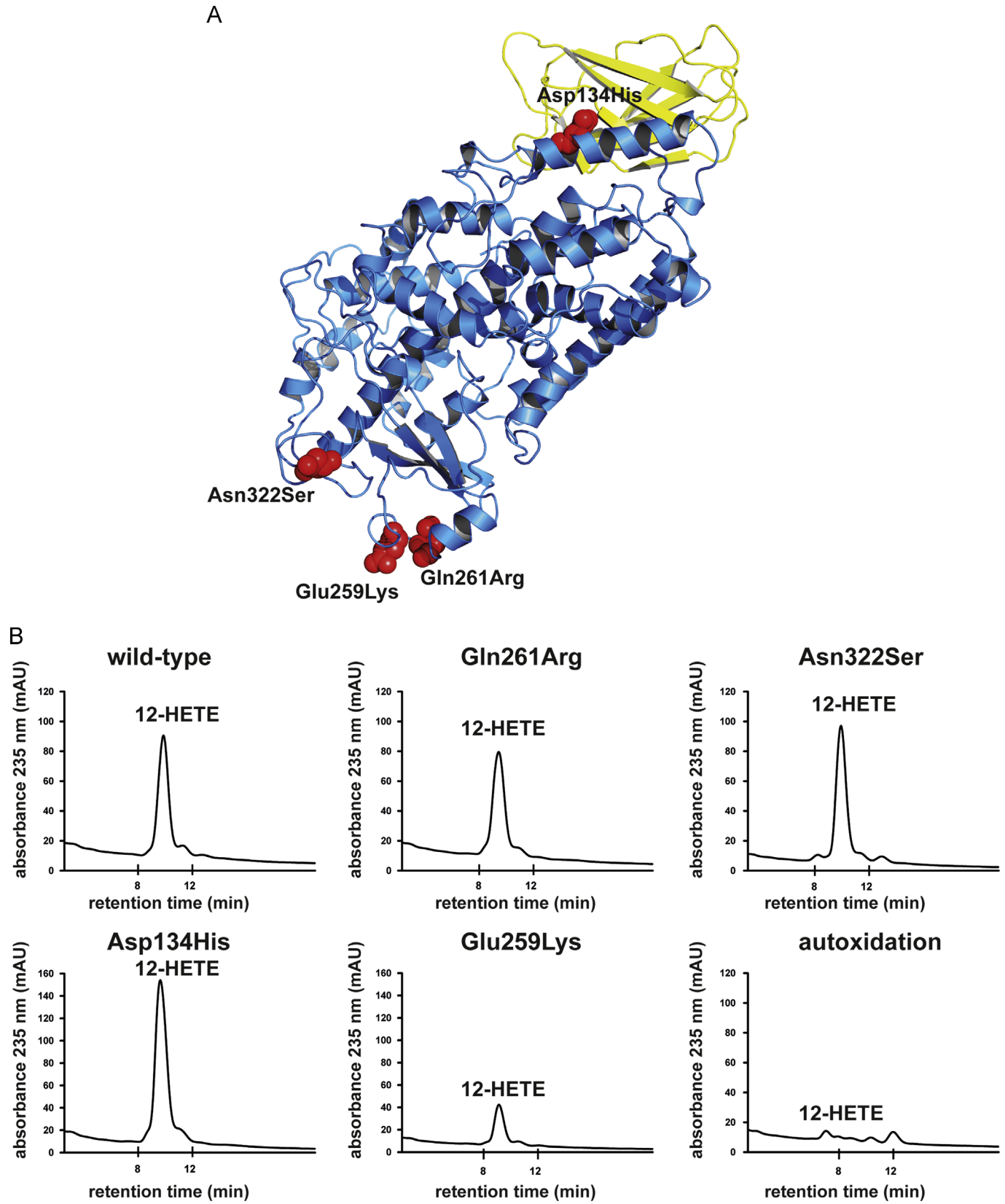

Fig. 3. Genetic variations in human ALOX12 (A) Structural localization of analyzed hALOX12 variations (hALOX12 model) N-terminal β-barrel domain is shown as yellow cartoon, the catalytic domain is represented as marine blue cartoon. The analyzed amino acid variations are shown as red spheres. (B) Reverse-phase HPLC chromatogram of hALOX12 wildtype and different genetic variations. The RP-HPLC chromatograms show that the wildtype and the hALOX12 variations are 12-lipoxygenating enzymes.

mutants (Table 7) indicating that the mutations had only minor effects on substrate binding and oxygenation rate. There are four premature stop variations described (Table 1) in the 1000 Genome database but neither of them has a global allele frequency of > 1%.

In hALOX15B the catalytic iron is coordinated by His373, His378, His553, Ser557, and the C-terminal Ile676. Interestingly, Ile676Val is a SNP with a global allele frequency of 9.6%, but our activity data indicated the lack of functional consequences for the amino acid exchange (Fig. 4C.). hALOX15B exhibits a single position specificity, as it converts arachidonic acid almost exclusively to 15-H(p)ETE [53]. The ortholog enzyme of mice (Alox15b) is an 8-lipoxygenating enzyme, and two amino acids Tyr603 and His604 are responsible for this functional difference [54]. In hALOX15B Asp603 and Val604 are at this position and previous mutagenesis

Table 5
Relative catalytic activities and product specificities of different hALOX12 variations.

hALOX12	variation ID	global frequency (%)	12-HETE (%)	rel. activity (%)
wildtype			> 99	100 ± 23
Asp134His	rs114985038	0.4	> 99	109 ± 36
Glu259Lys	rs4987104	0.0	> 99	60 ± 18
Gln261Arg	rs1126667	38.8	> 99	91 ± 10
Asn322Ser	rs434473	34.4	> 99	101 ± 23

Recombinant expression of hALOX12 and product HPLC analysis was performed as described in Materials and Methods with crude bacterial lysate supernatant of 5 ml bacterial cultures (n=5–10 for each variation). LOX content was determined by western blot analysis and HETE formation was correlated to the amount of recombinant protein. The amounts of HETE isomers were analyzed for each sample by HPLC and are used to calculate the catalytic activity of different enzyme species. The HETE formation of the wildtype was set to 100%.

Table 6
Relative catalytic activities and melting points of different hALOX15B variations.

hALOX15B	Variation ID	Global frequency (%)	15-HETE (%)	Rel. activity (%)	Melting temperature T_m (°C)
wildtype			> 99	100 ± 2	57.4 ± 0.2
Arg486His	rs9895916	8.7	> 99	100 ± 2	56.6 ± 0.3
Gln656Arg	rs28395869	38.6	> 99	104 ± 1	56.9 ± 0.2
Ile676Val	rs7225107	10.3	> 99	94 ± 7	55.0 ± 0.3
Ala416Asp	rs140152561	0.1	n.d.	n.d.	n.d.

Purified recombinant hALOX15B wildtype and the different enzyme variations were incubated with arachidonic acid as described in Material and Methods (n=4). The amounts for 15-HETE were quantified for each sample, and wildtype 15-HETE formation was set to 100%. Thermal shift assays were performed as described in Material and Methods. The inflection point of the first negative derivative represents the melting temperature (T_m) of the enzyme variation.

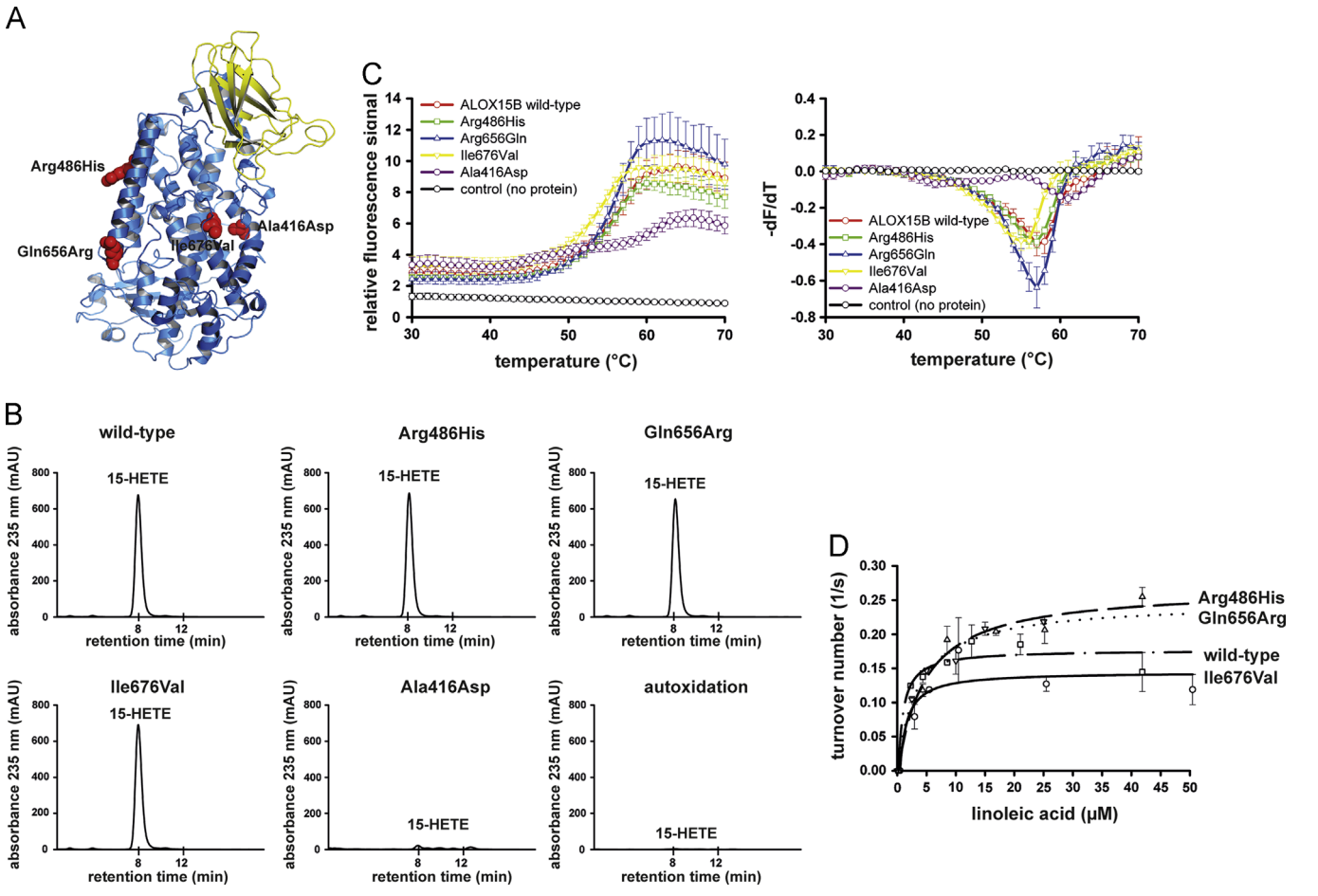

Fig. 4. Genetic variations in human ALOX15B (A) Structural localization of analyzed hALOX15B variations (PMDB: PM0078035) N-terminal β-barrel domain is shown as yellow cartoon; the catalytic domain is represented as marine blue cartoon. The amino acid variations are shown as red spheres. (B) Reverse-phase HPLC chromatogram of hALOX15B wildtype and different genetic variations. The RP-HPLC chromatograms show that the wildtype enzyme and the Arg486His, Gln656Arg, Ile676Val variants are 15-lipoxygenating enzymes, whereas the Ala416Asp mutant is catalytically silent. The small peaks in the HPLC profile originate from arachidonic acid autoxidation. (C) Thermostability of hALOX15B wildtype and different genetic variations using a fluorescent-based thermodynamic shift assay. The thermal denaturation curve of the Arg486His, Gln656Arg, and Ile676Val are similar to the wildtype enzyme, but the profile for the Ala416Asp variant is different indicating structural differences. The negative first derivative is calculated to determine the inflection point (T_m) of the unfolding curve more accurately. (D) Michaelis-Menten kinetics of hALOX15B wildtype and different genetic variations. All analyzed enzyme species follow Michaelis-Menten kinetics.

Table 7
Kinetic parameters of different hALOX15B variations.

hALOX15B	variation ID	k_{cat} (s^{-1})	K_m	R^2
wildtype	–	0.18 ± 0.01	1.2 ± 1.2	0.82
Arg486His	rs9895916	0.27 ± 0.02	2.8 ± 0.8	0.98
Gln656Arg	rs28395869	0.25 ± 0.02	3.7 ± 0.2	0.98
Ile676Val	rs7225107	0.12 ± 0.01	1.0 ± 0.6	0.93

Purified recombinant hALOX15B wildtype and different variations were incubated with linoleic acid as described in Material and Methods ($n=3$). The formation of conjugated dienes was followed by recording the increase in absorbance at 235 nm for 120 s. The resulting turnover rates (k_{cat}) and Michaelis constants (K_m) are given. The *R* square value (R^2) represents the accuracy of the hyperbolic equation.

studies (Tyr603Asp+His604Val) converted the mice 8-LOX to a 15-lipoxygenating enzyme. In the 1000 Genome database there is a rare natural occurring variant Val603Ile (rs192653434) with a global frequency of 0.1% at this position (Table 1) but the functional consequences of this amino acid exchange have not been studied. One might assume that an Ile-to-Val exchange represents a conservative mutation and therefore only minor functional effects should be expected. hALOX15B carries an Ala at the critical enantioselectivity determinant and therefore it has been classified as S-LOX [41–42]. Interestingly, there exists a rare genetic variation at amino acid position 416 (rs140152561), which leads to an Ala-to-Asp exchange. *In vitro*, this mutation causes inactivity of the enzyme **(**Fig. 4**B)** and thermal denaturation curve of this mutant strongly differs from those of the functional active SNPs and the wildtype enzyme **(**Fig. 4**C)** suggesting that the 3D-structure of the Ala416Asp mutant is severely hampered. To the best of our knowledge this is the first hALOX15B loss-of-function variation that has been published so far. Nevertheless its allele frequency is very low and therefore homozygous carriers of this mutation cannot be expected **(**Table S1**.)**.

ALOX12B (human 12-lipoxygenase, 12R type)

This LOX isoform consists of 701 amino acids and was first described in mouse and human skin [55–57]. Sixty nine non-synonymous coding variations have been published in the 1000 Genome database. There are no variations in the database leading to premature stop codons. Since recombinant expression of this particular LOX isoform in bacteria was not successful we were not able to test the functional consequences of the amino acid exchanges. Thus, we judged the probability of functional alterations on the basis of the character of the mutations. Multiple amino acid sequence alignments of many LOXs (see supporting information) suggested that the catalytic iron in hALOX12B is coordinated by His398, His403, His578, Asn582, and the C-terminal Ile701 (Table 1). There is a genetic variation (rs140063508) leading to a His398Tyr exchange but since Tyr in principle may also function as iron ligand via its phenolic OH-group we do not expect functional consequences for this amino acid exchange. A multiple sequence alignment with sequences of 153 LOX enzymes in different species indicated that the first three iron coordination ligands seem to be invariable (His) throughout the LOX enzyme family (data not shown), while the fourth (His, Asn, Ser, or Gly) and the C-terminal ligand (Ile, Val) are variable. The previous finding that Ser, which also contains a hydroxyl group, can function as an iron coordination ligand supports the idea that a His-to-Tyr exchange might only have minor functional effects. Searching the 1000 Genome database we did not find genetic variations for the triad residues or for the enantioselectivity determinants.

hALOX12B knockout mice show a strong phenotype since homozygous newborn die because of dehydration several hours after delivery [58]. In humans, genetic variants in the hALOX12B gene have been related to the development of autosomal recessive congenital ichthyosis (ARCI) [25]. Some of these mutants have been picked up in human 1000 genome project (Table S1), but except for Pro127Ser (rs72842957), which has a global frequency of 1.1%, the others must be classified as rare enzyme mutations. These data are consistent with the fact that ARCI is a rare skin disease (1:200,000-300,000 Europeans and Northern Americans). Unfortunately, the Pro127Ser variant is not expressed in HEK 293 cells and thus, no functional data are currently available [25]. If this variation with its relatively high global allele frequency of > 1% would be a strong inducer of ARCI a much higher prevalence of this skin disease should be expected [25].

ALOXE3 (epidermis-type lipoxygenase 3)

This LOX isoform was also discovered in mouse and human skin [59–60]. It has a length of 711 amino acids and a molecular weight of 80.6 kDa. Eighty three non-synonymous coding variations have been published in the human 1000 genome database (Table 1), but only two of them (Ile515Val, rs3027205; Arg678Cys, rs143246503) have a global frequency > 1% (3.0% and 1.9%, respectively). Ile515-Val is a conserved amino acid exchange while Arg678Cys is not conserved but localized on the enzyme surface far from the active site and therefore for both SNPs only minor functional consequences can be predicted (see supplement S3.).

As shown for hALOX12B variations in the hALOXE3 gene have been associated with ARCI [25] and some of them were also found in the 1000 Genome database (Table 1). For instance, the Ile515Val exchange was found in ichthyosis patients, but it remains unclear whether this mutation really impacts the functionality of the enzyme [25]. If this genetic variation would be the major cause of ARCI a much higher prevalence of this disease should be expected.

Searching in the 1000 Genome database we did not find any genetic variation leading to the introduction of a premature stop codon.

In comparison to other lipoxygenases which have a dioxygenase activity, hALOXE3 is mainly a hydroperoxide isomerase converting hydroperoxides (HpETEs) to specific epoxyalcohols [61], although it can also act as a dioxygenase after hydroperoxide activation [62].

hALOXE3 carries an Ala at the critical enantioselectivity position, and therefore it can be classified as S-LOX. This particular enzyme seems to follow the Coffa/Brash principle since the Ala-to-Gly exchange increases the R-product formation [63]. However, the enzyme does not follow the triad concept. Neither the potential iron ligands nor the enantioselectivity determinant is affected by any genetic variation.

Discussion

LOXs are lipid peroxidizing enzymes and their intracellular activity contributes to the regulation of the cellular redox homeostasis [2–3]. Since the cellular redox state is an important regulator of the gene expression pattern, SNP-induced dysfunctionality of LOX isoforms is likely to alter the cellular phenotype. To quantify the probability of genetically caused LOX dysfunction we screened the 1000 Genome database for all six human LOX genes and detected more than 450 non-synonymous coding variations (www.1000genomes.org). However, only 14 of them can be classified as SNPs (global allele frequency > 1%) while variations with a lower frequency must be classified as rare enzyme mutations. Functional analysis of eight SNPs throughout the hALOX family showed that except from the previously characterized hALOX15 near-null SNP Thr560Met (rs34210653) all other hALOX

SNPs had similar enzymatic properties (positional specificity, catalytic activity, thermostability) as the corresponding wildtype enzymes. Structural analysis showed that most of the analyzed SNPs are located at the enzyme surface, and therefore only minor effects on enzyme functionality have been predicted (Figs.1–4).

Most of the LOXs carry a catalytic iron at the active site and the C-terminal Ile is required for metal ion coordination in the active site [1,4]. Thus, all genetic variations leading to the introduction of premature stop codons are loss-of-function mutations. So far, 19 nonsense variations have been detected in all six hALOX genes, but neither of them has a global allele frequency $> 0.1\%$ indicating that all are rare enzyme mutations.

We found that almost all published hALOX loss-of-function variations have a global allele frequency of $< 0.1\%$. In contrast, all more common variations (SNPs) were without major functional consequences. This finding is consistent with a conclusion drawn previously, that rare SNPs and mutations (global allele frequency $< 5\%$) have a higher tendency to be related to a certain disease and it was suggested that future studies should focus more on them [28,64]. A major problem with such rare genetic variations is that they are often only discovered once or twice in large human populations and their observation is often restricted to one ethnic subpopulation [28]. Some of such subpopulations have not been adequately considered when the human 1000 Genome project was structured [29]. Thus, larger genome studies with up to 10,000 and more people and a broader spectrum of different ethnic backgrounds, especially in the Asian population, are required to determine the genetic diversity of rare genetic variations in the human population.

We observed that most of the functionally important amino acid residues in the six LOX genes are not affected by genetic variations. Thus, one might conclude that there is an evolutionary pressure on these residues to keep the functionality of these enzymes. Similar predictions were made for other enzymes evaluating the data from the human 1000 Genome project [28,59]. Although the exact biological role of the six LOX isoforms is not clear at the moment, these enzymes are well-conserved in two of the three major domains of terrestrial life, which indicates a biological role in living organisms [4]. Mammalian ALOX5 catalyzes the rate-limiting step in the biosynthesis of pro-inflammatory leukotrienes (LTs), which have been implicated in the pathogenesis of different inflammatory diseases [1]. Epidermal LOX isoforms (ALOX12B, ALOXE3) appear to be essential for the epidermal barrier formation and for skin development [25,57]. hALOX15 has been implicated in the synthesis of atheroprotective lipoxins and resolvins [65] and heterozygous carriers of the Thr560Met allele (rs34210653; global allele frequency 1.5%) have an increased risk in developing CAD [22]. We recently reported that the hALOX15 SNP rs61099320, which represents a Gly422Glu exchange, leads to a near-null variation of this enzyme which is related to protein misfolding [24].

Most of the detected genetic variations in LOXs encode for surface exposed amino acids. Since the active site of the enzyme is buried deeply inside the protein, surface exposed mutations are less likely to have functional consequences. However, since LOXs might function as dimers, surface exposed variations might well be of functional importance. [66]. A non-synonymous coding SNP in the human dopamine β-Hydroxylase gene showed that it prevents the tetramerization of the enzyme which is essential for its functionality [67]. Alternatively, SNPs at the protein surface might be important for the intracellular interaction with other regulatory proteins [68], but both aspects were not addressed by our experimental approach. Moreover, non-synonymous coding SNPs may be of a biological relevance since the binding of regulatory proteins during transcription, translation and mRNA processing (splicing) might be affected by such genetic variations [69–70]. To study the functional consequences of such non-synonymous coding variations on the DNA- or RNA level a completely different methodology must be applied.

Acknowledgment

We gratefully thank Deutsche Forschungsgemeinschaft (DFG) grant GRK1673/1 and CSIR (India) for funding this research project. We also thank Dagmar Heydeck for proof-reading of the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2013.11.001.

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