PRIMARY DETERMINANTS OF REACTION SPECIFICITY OF MURINE 12(R)-LIPOXYGENASE: SITE-DIRECTED MUTAGENESIS STUDIES

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

M. N. SUNITHA

Department of Animal Sciences
School of Life Sciences
University of Hyderabad
Hyderabad-500 046, INDIA

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I dedicate my thesis

to my parents,

Mrs. M. Omkara Devi & Prof. M. Kodanda Rao

UNIVERSITY OF HYDERABAD
(A Central University Established in 1974 by an act of Parliament)
Hyderabad- 500046, INDIA

DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Primary Determinants of Reaction Specificity of Murine 12(R)-Lipoxygenase: Site-directed Mutagenesis Studies"** has been carried out by me under the supervision of Prof. Reddanna and that this work has not been submitted for any degree or diploma of any other University earlier.

(Prof. P. Reddanna)
Research Supervisor

(M. N. Sunitha)
Research Student

UNIVERSITY OF HYDERABAD

(A Central University Established in 1974 by an act of Parliament)

Hyderabad- 500046, INDIA

CERTIFICATE

This is to certify that Ms. M. N. Sunitha has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the PhD ordinance of this University. We recommend her thesis **"Primary Determinants of Reaction Specificity of Murine 12(R)-Lipoxygenase: Site-directed Mutagenesis Studies"** for submission for the degree of Doctor of Philosophy of this University.

Prof. P. Reddanna
(Supervisor) _____

Head
Department of Animal Sciences _____

Dean
School of Life Sciences _____

CONTENTS

ABBREVIATIONS

15-H(p)ETE	(5Z, 8Z, 11Z, 13E)-15-Hydro (pero)xyeicosa-5,8,11,13-tetraenoic acid
12-H(p)ETE	(5Z, 8Z, 10E, 14Z)-12-Hydro (pero) xyeicosa-5,8,10,14-tetraenoic acid
11-H(p)ETE	(5Z, 8Z, 12E, 13Z)-11-Hydro (pero) xyeicosa-5,8,12,13-tetraenoic acid
9-H(p)ETE	(5Z,7E,11Z,14Z)-9- Hydro (pero) xyeicosa-5,7,11,14-tetraenoic acid
8-H(p)ETE	(5Z,9E,11Z,14Z)-8-Hydro(pero)xyeicosa-5,9,11,14-tetraenoic acid
5-H(p)ETE	(6E,8Z,11Z,14Z)-5-Hydro(pero)xyeicosa-6,8,11,14-tetraenoic acid
13-H(p)ODE	(9Z,11E)-13-Hydro(pero)xyoctadeca-9,11-dienoic acid
9-H(p)ODE	(10E,12Z)-9-Hydro(pero)xyoctadeca-10,12-dienoic acid
Amp	Ampicillin
BSA	Bovine Serum Albumin
CP-HPLC	Chiral phase HPLC
DiH (p) ETE	Dihydro (pero) xyeicosatetraenoic acid
dNTP	Deoxynucleotide triphosphate
GC/MS	Gas chromatography/Mass spectrometry
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl-ß-thiogalactopyranoside
Kan	Kanamycin
kb	Kilo base
LB-Medium	Luria Bertani Medium
LT	Leukotriene
LX	Lipoxin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RP-HPLC	Reverse Phase HPLC
SP-HPLC	Straight Phase HPLC

Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Gln	Q	Glutamine
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

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INTRODUCTION

Arachidonic acid (5,8,11,14-eicosatetraenoic acid, 20:4) is a polyunsaturated fatty acid with four isolated *cis* double bonds. It is predominantly found esterified in membrane phospholipids in all mammalian cells. The high degree of unsaturation of polyenoic fatty acids confers structural flexibility to biomembranes keeping their lipid bilayers in a semi-liquid state, even at subzero temperatures. This membrane flexibility allows active and/or passive alterations of the cell shape, which is an important requirement for normal cell function. The double bonds of polyenoic fatty acids also provide the possibility to react with molecular dioxygen. Such oxygenation reactions may be catalyzed by transition metals (non-enzymatic) or by various types of oxygenases. In fact, for oxygenation of polyenoic fatty acids three major types of oxygenases have been identified: i) cyclooxygenases (COXs), ii) lipoxygenases (LOXs), and iii) cytochrome P-450 isozymes (Brash, 2001). The products of enzymatic and non-enzymatic lipid peroxidation are potent regulatory substances, which are synthesized intracellularly and then released into the extracellular space to act locally (local hormones or autocoids) or to be transported with the blood to other tissue and organs. They regulate many cell functions and play crucial roles in a variety of physiological and pathophysiological processes, such as inflammation, regulation of vasodilation, vascular permeability, pain, recruitment of leukocytes and others. These products are termed as 'eicosanoids' as most of them are derived from C_{20} (eicosa) polyenoic fatty acids. The structures and functions of these oxygenated derivatives are quiet diverse. The critical differences in the biological properties of these signal transducers are the number, the position and the stereochemistry of the oxygen moiety and these parameters determine the information content of eicosanoids. Thus, in the world of eicosanoids, oxygen is the currency of information. How cells and tissues regulate the balance of these mediators and the biological properties of these molecules is a rewarding area of investigation today and also for the foreseeable future (Soberman, 2003).

Fig. 1. Chemical structure of arachidonic acid

Formula: C_{20} O_2 H_{32}, double bonds between C_{5-6}, C_{8-9}, C_{11-12}, C_{14-15}

1.1 ARACHIDONIC ACID CASCADE:

The rate-limiting step in eicosanoid biosynthesis is the release of free arachidonic acid from membrane phospholipids. The bulk of arachidonic acid in resting cells is esterified to the sn-2 position of membrane ester lipids. Although multiple enzymes can catalyze hydrolysis of the ester bonds to produce free arachidonic acid, the dominant pathway in most mammalian cells is *via* the activity of a phospholipase A_2 (PLA_2) (Smith et al., 1992). The cellular levels of free arachidonic acid are normally very low since liberated fatty acids are rapidly further metabolized. Free arachidonic acid constitutes the substrate for three distinct oxidative pathways (Fig. 2):

(i) The cyclooxygenase (COX) pathway, which leads to the formation of cyclisation products of arachidonic acid that include prostaglandins, prostacyclins and thromboxanes. The cyclooxygenase is a bifunctional enzyme and exhibits cyclooxygenase and peroxidase activity. The cyclooxygenase activity introduces two molecules of oxygen into arachidonic acid to form the cyclic hydroperoxy endoperoxide PGG_2, which is subsequently reduced by the peroxidase activity of the enzyme to the hydroxy endoperoxide, PGH_2 (Smith et al., 1991). PGH_2 constitutes substrate for further enzymatic modifications leading to the formation of the prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$,), prostacyclin (PGI_2) or thromboxane A_2 (TxA_2) (Smith et al., 1992). COX-isoforms employ their heme moiety to generate a tyrosyl radical that abstracts hydrogen from C-11 of arachidonic acid. The two COX-

isoforms are the target of most prostaglandin synthesis inhibitors, particularly of the nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin (Levy, 1997).

(ii) The lipoxygenases (LOX) pathway, which leads to the formation of linear eicosanoids containing conjugated double bond systems. LOXs produce fatty acid hydroperoxides that are rapidly reduced to the corresponding hydroxy compounds by glutathione peroxidases. LOXs contain one mole non-heme iron per mole enzyme and this transition metal is involved in the rate-limiting step of the LOX reaction, the initial hydrogen abstraction. Following hydrogen abstraction LOXs catalyze the introduction of atmospheric oxygen into the fatty acid chain and it depends on the positional specificity of the enzyme, at which carbon atom oxygen is inserted. (Kühn et al., 1986; Yamamoto et al., 1992). Hence, 5-LOXs generate 5-HpETE (5-hydroperoxyeicosa-6,8,11,14-tetraenoic acid), 12-LOXs generate 12-HpETE (12S-hydroperoxyeicosa-5,8,10,14-tetraenoic acid) and 15-LOXs generate 15-HpETE (15-hydroperoxyeicosa-5,8,11,13-tetraenoic acid). 5- and 15-HpETE can be further metabolized to epoxy leukotrienes. 5,6-epoxy leukotriene A_4 (5,6-LTA_4) constitutes the substrate for the cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4), the mixture of which is known as slow-reacting substances of anaphylaxis (Lewis et al., 1980).

(iii) Cytochrome P-450 pathway, which leads to the formation of two principle metabolites. i) monohydroxylated eicosanoids, most of which do not contain conjugated double bond systems (20-HETE, 19-HETE, 18-HETE etc). ii) epoxy fatty acids, which originate from epoxidation of double bonds (5,6-ETE, 8,9-ETE, 11,12-ETE, 14,15-ETE). In contrast to the LOX reaction cytochrome P-450-catalysed oxygenation involves insertion of atomic oxygen. In fact, molecular dioxygen is split into two oxygen atoms, one of which is introduced into the fatty acid substrate and the other one is reduced to water (Capdevila et al., 1992). Epoxy eicosanoids are rapidly hydrolyzed to

dihydroxylated fatty acids. In tissues like kidney and cornea, cytochrome P-450 mediated oxygenation of fatty acids results in the formation of biologically active products and plays an important role in the physiology of these tissues.

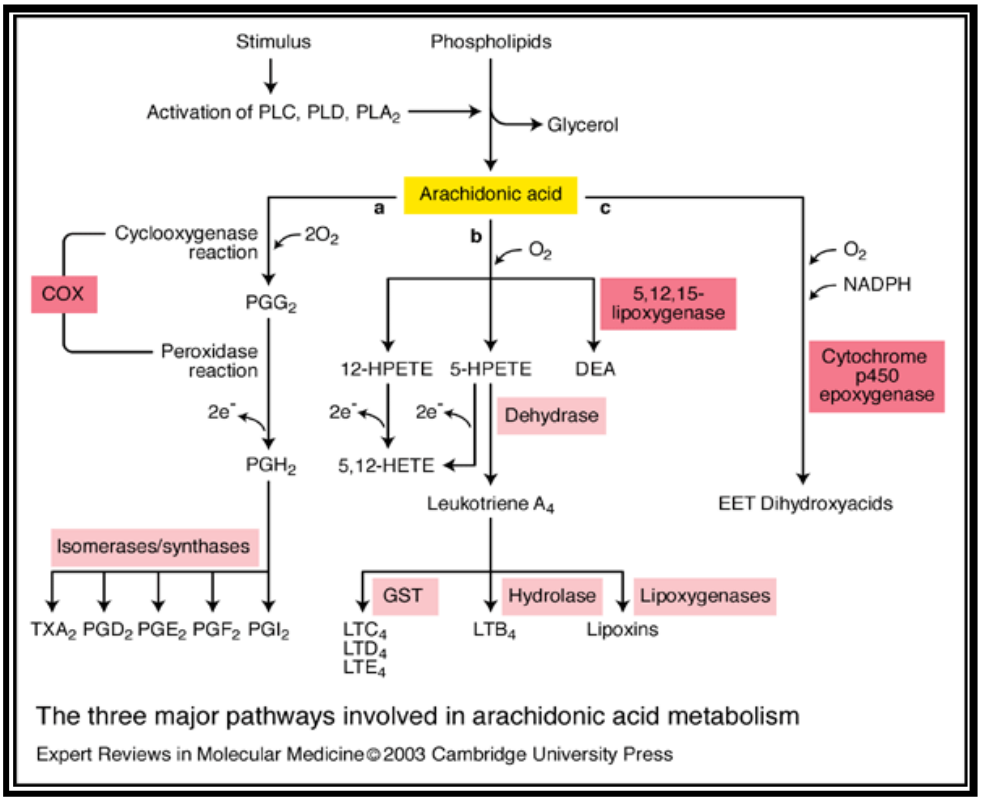


Figure 2. The three major pathways involved in oxidative arachidonic acid metabolism.. Arachidonic acid can be released by three independent pathways, of which direct action of PLA_2 on a phospholipid is most dominant. The three oxidative pathways are (a) The cyclooxygenase (COX) pathway results in the formation of prostaglandin G_2 (PGG_2) from arachidonic acid by a cyclooxygenase reaction (b) The lipoxygenase pathway forms hydroperoxyeicosatetraenoic acids (HPETEs) and dihydroxyeicosatetraenoic acid (DEA) by lipoxygenase and subsequently converts these to hydroxyeicosatetraenoic acids (HETEs) by peroxidases, leukotrienes (e.g. LTC_4) by hydrase and glutathione *S*-transferase (GST), and lipoxins by lipoxygenases. (c) The epoxygenase pathway forms epoxyeicosatrienoic acid (EET) and dihydroxyacids by cytochrome P450 epoxygenase.

COXs and LOXs initiate the oxygenation reaction by activating the fatty acid substrate. This activation is accomplished by catalyzing hydrogen abstraction from a bisallylic methylene, which leads to the formation of a

carbon centered radical (allylic or pentadienylic) with multiple reactive centers for addition of molecular oxygen. The regiochemistry of the oxygenation in these transformations is governed by the geometry of the enzyme active site and possibly by control of the protein over the spin density distribution in the delocalized radical. In contrast, the cytochrome P-450-catalysed oxygenation involves activation of the second substrate, the atmospheric dioxygen, destabilizing the intramolecular O-O bond.

1.2. CYCLOOXYGENASE PATHWAY

1.2.1 Cyclooxygenase reaction:

Prostaglandin H synthase (PGHS) catalyzes the conversion of arachidonic acid (AA) to prostaglandin H_2 (PGH_2) creating five new chiral centers. PGHS is a bifunctional enzyme that first converts AA into PGG_2 in the cyclooxygenase reaction, followed by reduction of the hydroperoxide via its hydroperoxidase activity.

Two COX-isozymes (COX-1 and COX-2) exist in mammals, which share 60% sequence identity (Marnett et al., 1999; Hla et al., 1999) but both proteins catalyze identical reactions. In a somewhat simplified picture, COX-1 is constitutively expressed, whereas COX-2 expression is induced in response to pro-inflammatory stimuli. Thus COX-2 constitutes a pharmaceutical target for anti-inflammatory drugs. Recent studies have shown that endocannabinoids comprising amide derivatives of AA are suitable substrates for COX-2 but not COX-1 (Kozak et al., 2000).

Arachidonic acid, that contains four double bonds, is converted *via* the COX pathway to the prostaglandins of the 2-series (PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, TXA_2) (Fig. 3). These compounds differ from each other with respect to the chemical nature of the substituents at the prostane ring and in the stereochemistry of these ligands. The unstable endoperoxide PGH_2 formed via the COX reaction can alternatively be converted into classical prostaglandins,

prostacyclin (PGI_2) or thromboxane (TXA_2). In mammals, TXA_2, is mainly produced by activated blood platelets and induces vasoconstriction, cell adhesion to the vessel wall and platelet aggregation (Spagnuolo et al., 1980). PGI_2 is formed by vascular endothelial cells and antagonizes the TXA_2-induced effects. Thus, the TXA_2-PGI_2 steady state appears to be important for systemic blood pressure regulation and for the pathogenesis of thrombosis.

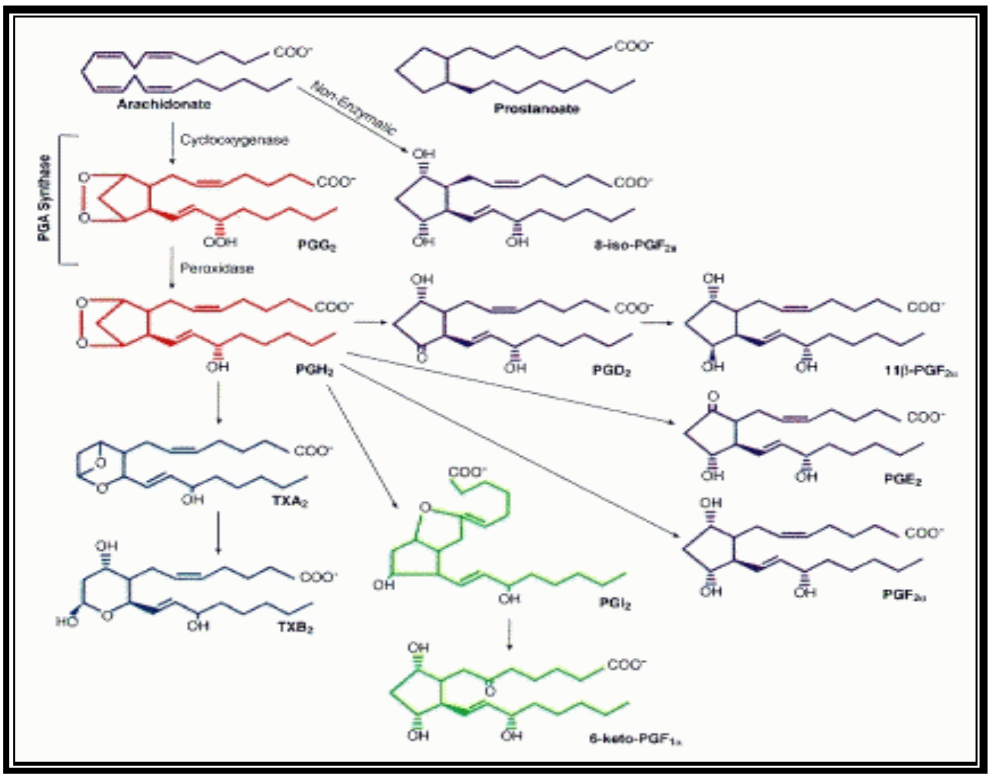


Fig. 3. Cyclooxygenase pathway of the arachidonic acid cascade: Cyclooxygenase (COX) enzymes, also referred to as prostaglandin H synthases or prostaglandin endoperoxide synthases, catalyze the rate limiting steps in prostaglandin (PG) and thromboxane (TX) synthesis. The first step in the catalysis involves addition of O_2 atoms to C-11 and C-15 to yield PGG_2. The 15-hydroperoxide group of PGG_2 is then converted to an alcohol forming PGH_2 by the peroxidase activity of the enzyme. PGH_2 is subsequently converted to other PGs (PGD_2, PGE_2, $PGF_{2?}$, PGI_2) or thromboxanes (TXA_2) by specific cellular synthases.

1.2.2. COX-1 and COX-2:

Two distinct COX isoenzymes exist in mammals. COX-1 was first purified and characterized in the 1976 (Miyamoto et al., 1976; Hemler and

Lands, 1976) and the gene was isolated in 1988 (DeWitt and Smith, 1988; Yokoyama et al., 1988). The discovery and cloning of the second COX isoenzyme, COX-2, in 1991 (Xie et al., 1991; Kujubu et al., 1991) has revolutionized the understanding of PGs and their functions in normal physiology and in disease. The two COX-isoforms share a high degree of structural and functional similarity but are under the control of different regulatory elements. Both enzymes are homodimers with a molecular weight of 140 kDa and contain one heme-moiety per monomer (Garavito et al., 2002). They share 63% identity at the amino acid level and their 3D-structures are superimposable (Luong et al., 1996; Kurumbail et al., 1996). COX-2 contains an 18 amino acid insertion in its carboxyl terminal region while COX-1 has 8 extra amino acids at the N-terminus of the mature protein (Smith and DeWitt, 1996). Both proteins share three conserved N-linked glycosylation sites and COX-2 contains an additional gylcosylation site within the extra stretch of 18 amino acid (Otto et al., 1993).

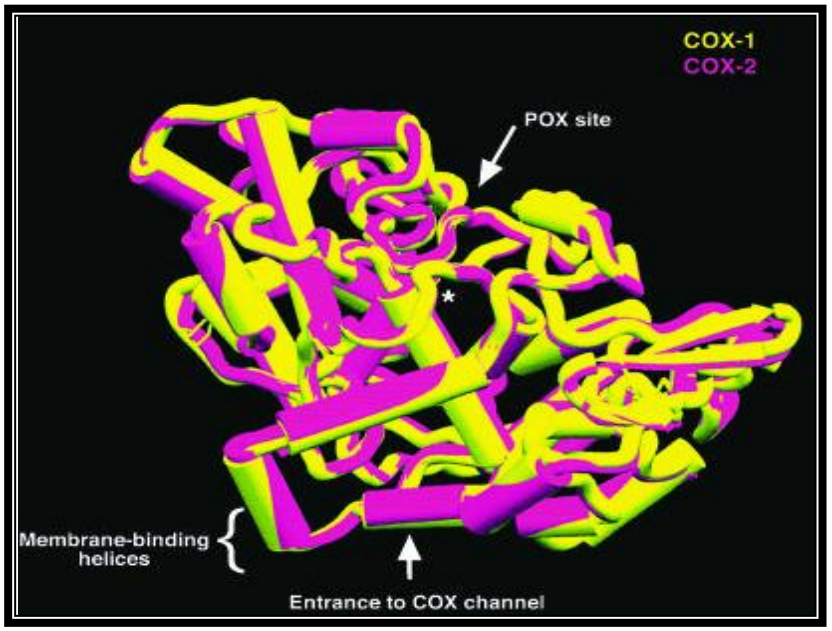


Fig. 4: The COX-1 and COX-2 backbones, overlaid COX-1 is shown in yellow and COX-2 in pink. The two structures are almost perfectly superimposable. The amphipathic helices that form the site of monotopic membrane attachment are indicated. The peroxidase (POX) active site lies on the opposite side of the molecule from the entrance to the COX active site. The actual position of the COX active center is marked by the asterix, found near the center of the molecule.

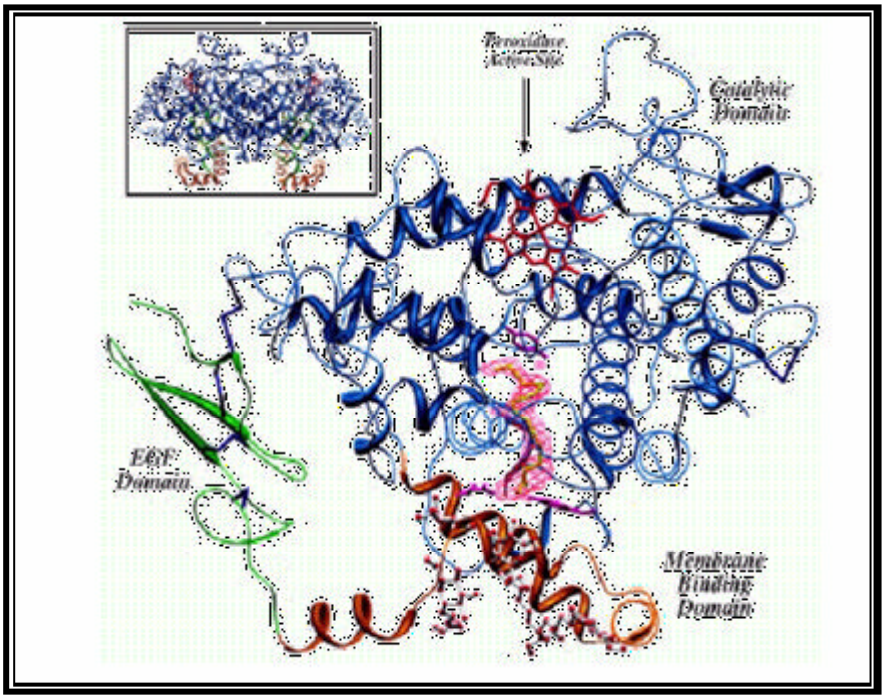


Fig. 5: The X-Ray Structure of Cyclooxygenase (COX-1). The figure shows three distinct domains. 1. EGF domain, 2. Membrane binding domain 3.Catalytic domain.

The mature COX proteins contain three distinct domains (Luong et al., 1996; Kurumbail et al., 1996). The first structural motif resembles the epidermal growth factor (EGF) and thus, is termed the EGF-like domain. The function of this domain in COX enzymes is poorly understood but it appears to be important for associating the enzyme to the lipid bilayer of biomembranes (Garavito et al., 1994; Picot et al 1994). The second domain contains a series of amphipathic helices, which form the membrane-binding domain (MBD). Unlike integral membrane proteins, which usually contain membrane-spanning helices, COX-isoforms are only attached to the membrane surface. In fact, they associate with the membrane of the endoplasmic reticulum (ER) *via* hydrophobic interactions of surface exposed hydrophobic amino acids and thus, may be considered monotopic membrane proteins (Li et al., 1998). The third domain is a large globular region, which contains the centers for cyclooxygenase and peroxidase activity (Garavito et al., 1994). The COX active site is located in the middle of a narrow

hydrophobic channel (Seibold et al., 2000) framed by the membrane attachment helices, which allows free arachidonic acid to directly access from the lipid compartment of the membrane to enter the active site without being released into the aqueous environment of the cytosol (Loll et al., 1996).

The amino acids involved in substrate binding and catalysis are very similar among the two isoforms. Two important differences are found at residues 434 and 523 (COX-1 numbering) where isoleucine occupies each position in COX-1 but valine is present in COX-2 (Luong et al., 1996; Kurumbail et al., 1999). These amino acids are part of the substrate-binding channel and one of the major consequences of these substitutions is that the volume of the active site of COX-2 is bigger when compared with COX-1. This increased active site volume may be considered as structural reason for the broad substrate specificity of COX-2. Moreover, this size difference was used for the development of isoform-specific inhibitors for COX-1 and COX-2 (Gierse et al., 1996). In fact, mutating Ile523 in COX-1 to Val (increase in active site volume) renders it sensitive to COX-2-selective inhibitors (Wong et al., 1997). Other amino acid differences in this channel that also play a role in determining substrate and inhibitor specificity include His513 (COX-1) to Arg (COX-2), and Ser516 (COX-1) to Ala (COX-2).

1.2.3 Mechanism of COX inhibition:

NSAID binding to the COX active sites of COX-1 and -2 have been studied extensively, and a number of crystal structures of NSAID/COX complexes are currently available (Loll et al., 1995; Loll et al., 1996). All the classical NSAIDs inhibit both PGHS-1 and -2 but many tend to bind more tightly to PGHS-1 (Munroe 1995; DeWitt 1999). Based upon their inhibitory mechanisms COX inhibitors are grouped into four classes (Table 1). All but the first class are reversible inhibitors in that once the drug is removed, COX

activity is restored, albeit at different rates depending on the chemistry of the compound. The first class, which includes aspirin and recently developed COX-2-specific aspirin-like molecules irreversibly, inactivate COX activity by acetylating an active site serine. Aspirin is considered COX-1-selective since the doses required for effective COX-2 inhibition are 10- to 100-fold higher than those required for COX-1. Aspirin-acetylated COX-1 does not exhibit any catalytic activity any more enzymatic activity. In contrast, due to its larger substrate-binding channel, acetylated-COX-2 retains its peroxidase activity, and effectively converts arachidonic acid to 15R-HpETE in a LOX-like reaction.

Mode of Inhibition	Selectivity	Examples	Comments
Covalent Modification	COX-1 COX-2 (APHS)	Aspirin APHS*	Acetylation of active site serine
Reversible, Competitive Inhibition	COX-1 and 2	Ibuprofen Mefenamate	Compete with AA† for active site.
Slow, Time-dependent Inhibition	COX-1 and 2	Indomethacin Flurbiprofen	Salt bridge formation with Arg. 120
Time-dependent COX-2 Inhibition	COX-2	Celecoxib Rofecoxib SC58125	Larger side groups to occupy extra side pocket in COX-2

* o-(acetoxyphenyl)hept-2-ynyl sulfide
† arachidonic acid

Table 1: Mode of inhibition by COX inhibitors. The inhibitors are classified on their mode of inhibition towards COX-1 and COX-2 enzymes.

1.3 LIPOXYGENASE PATHWAY

1.3.1. Introduction:

Lipoxygenases (LOX) are dioxygenases, which catalyze the insertion of molecular oxygen into polyunsaturated fatty acids containing one or more

(1Z, 4Z)-penta-1, 4-dienoic structural units. The primary products formed are hydroperoxy fatty acids containing a Z, E-conjugated diene system. Lipoxygenases are a family of structurally related non-heme, iron-containing enzymes found ubiquitously in all plants and animal systems. They were also discovered in lower marine organisms, such as algae, sea urchin, starfish, surf clam, and corals (Hawkins et al., 1987, Brash et al., 1991, Hada et al., 1997 and Brash et al., 1996) as well as in fungi (Bisakowski et al., 1997; Su et al., 1998). They were thought to be absent in lower prokaryotes like bacteria as they do not have the essential fatty acid substrates, but recently LOX sequences have been detected in *P. aeruginosa* and *S. cellulosum* (Porta et al., 2001). Interestingly, judged from the amino acid sequences bacterial LOXs appear to be more closely related to mammalian enzymes than to plant LOXs. In fact, it was speculated that LOXs have been introduced into prokaryotes by horizontal transfer. Lipoxygenases were found to be absent in yeast as no gene was found in the yeast genome (Brash, 1999).

The LOX reaction is highly regio- and stereospecific and mammalian LOXs are classified as 5-, 8-, 12-, and 15-lipoxygenases depending on the position of oxygen insertion during arachidonic acid oxygenation. LOXs are single chain polypeptides with molecular masses between 70 - 100 kDa. They have been purified and characterized from both plant and animal tissues. The sequences of more than 50 LOXs have been reported and many of them have been expressed as active proteins in heterologuous recombinant expression systems. In general, plant enzymes have a higher molecular mass than mammalian LOXs (Prigge et al., 1997). Higher plants contain multiple lipoxygenases with at least eight isozymes identified in soybean, *Glycine max*. Soybean LOX-1 was one of the first enzymes to be cloned in 1987 (Shibata et al., 1987) and was expressed in the bacterial systems. Moreover, it is relatively easy to purify the enzyme from natural sources; it is sufficiently stable and can be obtained in large quantities. This LOX isoform usually serves as prototype for mechanistic studies on the LOX reaction. Given the similarities among

lipoxygenase sequences, it is expected that many but not all of the results obtained for soybean LOX-1 can be extended to other LOX isoforms.

1.3.2 Lipoxygenase reaction:

Most lipoxygenases contain one non-heme iron per mole enzyme. In soybean lipoxygenase, the iron is high spin (Gaffney, 1996) and exists as ferrous (Fe^{2+}, catalytically silent) or ferric (Fe^{3+}, active form) forms. In most proposed mechanisms, the iron shuttles between ferrous and ferric form during catalytic cycle.

The mechanism of the lipoxygenase reaction involves three consecutive steps:

(i) Stereo-selective hydrogen abstraction from a doubly allelic methylene group [(1Z, 4Z)-penta-1, 4-diene] forming a carbon centered fatty acid radical. The hydrogen abstraction is the rate-limiting step in the overall reaction. Hydrogen is abstracted as proton and the electron is picked up by the ferric lipoxygenase, which is reduced to the ferrous form.

(ii) Radical rearrangement ([+2] and [-2], Kühn et al., 1986) which is accompanied by a Z, E-diene conjugation.

(iii) Stereospecific (S- or R-) insertion of molecular dioxygen at C-1 or C-5 of the pentadienyl system forming an oxygen-centered fatty acid hydroperoxide radical. This radical intermediate is subsequently reduced to the corresponding anion and the enzyme is oxidized back to ferric form. From the stereochemical point of view each of these steps can proceed in different ways.

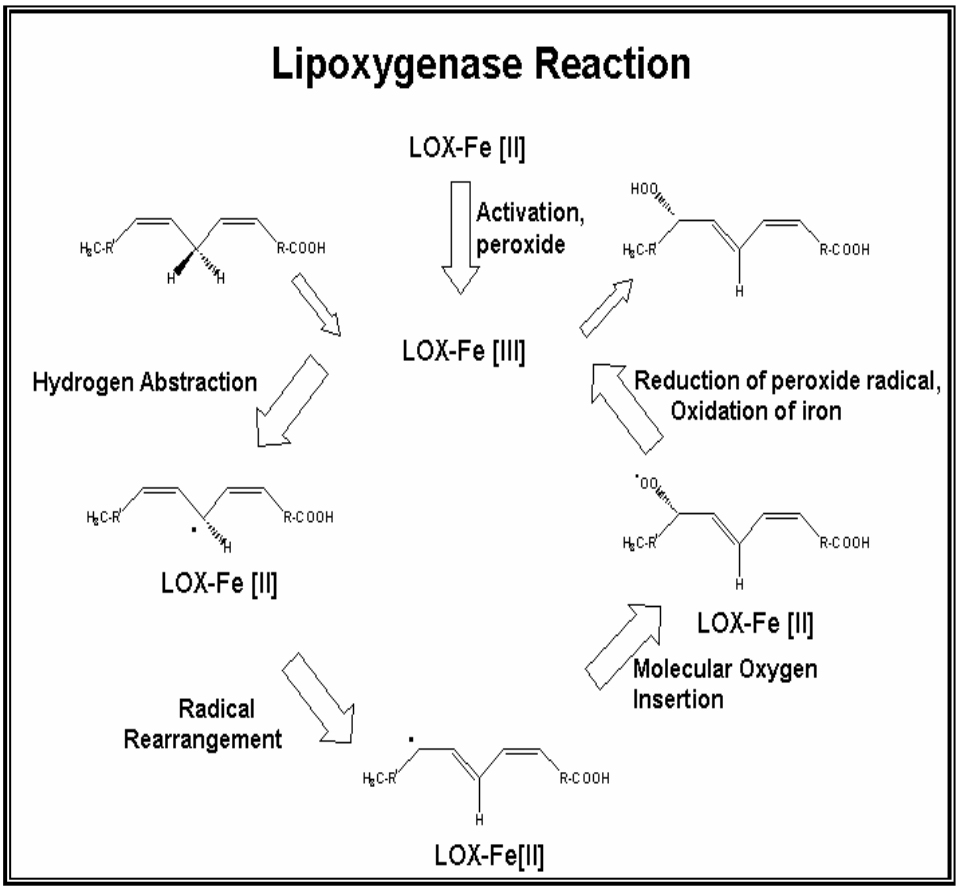


Fig. 6. Schematic representation of the catalytic cycle of LOX. LOX is usually in the inactive ferrous state. Oxidation to the active ferric state is required for catalysis. The abstraction of hydrogen from doubly allylic methylene leads to the formation of a pentadienyl radical and a proton. The remaining electron reduces iron to the ferrous state. Antarafacial insertion of molecular oxygen generates a hydroperoxide radical, which is reduced to the hydroperoxide anion by the simultaneous oxidation of iron to the ferric state. A proton is accepted to form the hydroperoxide. The ferric enzyme is then able to initiate a new reaction cycle

(i) Hydrogen abstraction:

Arachidonic acid contains three bisallylic methylenes, which are pro-chiral carbon atoms. Each of these pro-chiral centers carries two hydrogens but most LOX prefer to abstract only one of these hydrogens. Thus, the LOX reaction involves regio- and stereo-selective hydrogen abstraction and it depends on the reaction specificity of the enzyme and which hydrogen is removed.

(ii) Radical rearrangements:

The classical mechanism of the LOX reaction involves the formation of a pentadienyl radical. In this intermediate the density of the unpaired radical electron may be delocalised over the entire pentadienyl system, which yields a planar structure of this part of the substrate molecule. However, more recent structural data (Minor, 1996) suggest that the formation of a planar pentadienyl radical may be hindered sterically. Thus, the formation of an allyl radical was proposed, in which the radical electron may not be delocalised over the entire pentadienyl system but rather over a part of it. Recent spectroscopic studies on the interaction of the soybean LOX-1 with linoleic acid suggested the formation of a dioxygen-bridged allyl radical (Nelson, 1994). According to this hypothesis the direction of the radical rearrangement ([+2] or [-2]) depends on the conformation the fatty acid may adopt at the active site.

(iii) Oxygen insertion:

At the present time it is believed that LOXs may have neither a specific dioxygen-binding site nor a separate tunnel, *via* which the oxygen may penetrate into the active site. Instead, it has been suggested that oxygen insertion proceeds diffusion-controlled and that the gas may travel through the fatty acid binding cavity. If this is true additional assumptions must be made in order to explain the high degree of stereospecificity of most LOXs.

1.3.3 Historical Background:

In 1974 the first mammalian LOX (platelet-type 12-LOX) was discovered in human platelets (Hamberg and Samuelsson, 1974). Six months later another LOX-isoform (15-LOX) was described in rabbit reticulocyte, which was capable of oxidizing phospholipids and biomembranes (Schewe,

1975). This enzyme was purified to homogeneity from rabbit immature red blood cells and was well characterized with respect to its protein chemical and enzymatic properties (Rapaport et al., 1979). In 1988, a similar enzyme was purified from human eosinophils (Sigal et al., 1988) and comparison of its properties led to the conclusion that this enzyme was the human orthologue of the rabbit 15-LOX. The search for reticulocyte-type 15-LOXs in other mammalian species remained unsuccessful until now. However, in mice (Chen et al., 1994), pigs (Yoshimoto et al., 1991) and rats, a leukocyte-type 12-LOX was identified and several lines of experimental evidence suggest that these enzymes may be considered the functional equivalent of the reticulocyte-type 15-LOX in these species. The cDNA for the leukocyte-type 12*S*-lipoxygenase was first cloned from porcine leukocytes (Yoshimoto et al., 1990), and later from murine (Chen et al., 1994; Freiremoar et al., 1995), rat (Hada et al., 1994), bovine (De Marzo et al., 1992) and rabbit (Berger et al., 1998) sources. The cDNA for the platelet-type 12*S*-lipoxygenase cDNA was isolated from human (Funk, 1990; Yoshimoto, 1990) and murine tissues. The epidermis-type 12*S*-lipoxygenase cDNA was cloned from newborn mouse epidermis (Funk, 1996; Kinzig et al., 1997). The open reading frames of cDNAs for the three isozymes predict 662 amino acids and molecular weights of approximately 75,000 Da. When the amino acid sequences of the murine 12S-lipoxygenases were compared, the epidermal 12*S*-lipoxygenase displays 60% identity with both platelet and leukocyte isozymes (Chen et al., 1994). The cDNA of the murine platelet 12*S*-lipoxygenase is 58% identical to the leukocyte isozyme (Chen et al., 1994). As leukocyte-type 12*S*-lipoxygenases shows much higher identity (71–86%) to human and rabbit reticulocyte 15-lipoxygenases (named 15-lipoxygenase-1) they are collectively called 12/15-lipoxygenases (Sun and Funk, 1996). However, all 12*S*-lipoxygenases display only 40% identity to 5-lipoxygenase (Funk et al., 1996; Kühn et al., 1999), indicating that 12- and 5-lipoxygenases may belong to distinct enzyme subfamilies.

In 1997, a second type of 15-LOX was cloned from human hair roots (Brash et al., 1997). This enzyme, which was named epidermis-type 15-LOX or 15-LOX-2, only shares a low degree of sequence homology with the reticulocyte-type 15-LOX and strongly differs from the latter isoform with respect to its protein sequence and enzymatic properties. It is expressed in skin, prostate, lung, and cornea (Brash et al., 1997). The cDNA encodes for 676 amino acids and the predicted molecular weight was 76 kDa. When expressed in HEK 293 cells, the enzyme converts arachidonic acid exclusively to 15*S*-HPETE. In contrast to the reticulocyte-type 15-LOX, linoleic acid is less well metabolized. Although the biological role of this LOX isoform is far from clear, it may be related to skin functionality and prostate cancer. Interestingly, the orthologue murine enzyme is an arachidonate 8-LOX (Krieg et al., 1998) and a combined strategy of site directed mutagenesis and chimera formation indicated the structural basis for the difference in the positional specificity of the two isoforms (Jisaka et al., 2000).

The 8(*S*)-lipoxygenase cDNA was cloned from phorbol ester-treated mouse skin by RT-PCR strategies using degenerate primers based on highly conserved sequences in mammalian lipoxygenases and, subsequently, by screening skin-derived cDNA libraries. The open reading frame encodes a protein of 677 amino acids with a calculated molecular weight of 76.23 kDa (Jisaka et al., 1997; Krieg et al., 1998). 8(*S*)-Lipoxygenase is most closely related to human and bovine 15(*S*)-lipoxygenase-2 exhibiting 78% and 75% amino acid identity, respectively. Based on the high sequence identity and on the structure and chromosomal localization of the corresponding gene the human 15(*S*)-lipoxygenase-2 is considered orthologuous enzyme of murine 8(*S*)-lipoxygenase (Brash et al., 1997). Sequence comparison with other mammalian lipoxygenases shows about 54% amino acid identity with 12(*R*)-lipoxygenases, 50% with epidermis-type lipoxygenases-3, 43% with 5(*S*)-lipoxygenases, but only 38% identity with the 12(*S*)- and 12/15(*S*)-lipoxygenases. In a phylogenetic tree (Fürstenberger et al., 2002), which

summarizes LOX subfamilies and displays the relative evolutionary distances between the individual members of the mammalian lipoxygenases, the 8(*S*)-lipoxygenase and its human and bovine orthologues are grouped together with other skin-derived isoenzymes including 12(*R*)-lipoxygenases and epidermis-type lipoxygenases-3 in a distinct epidermis-type subfamily.

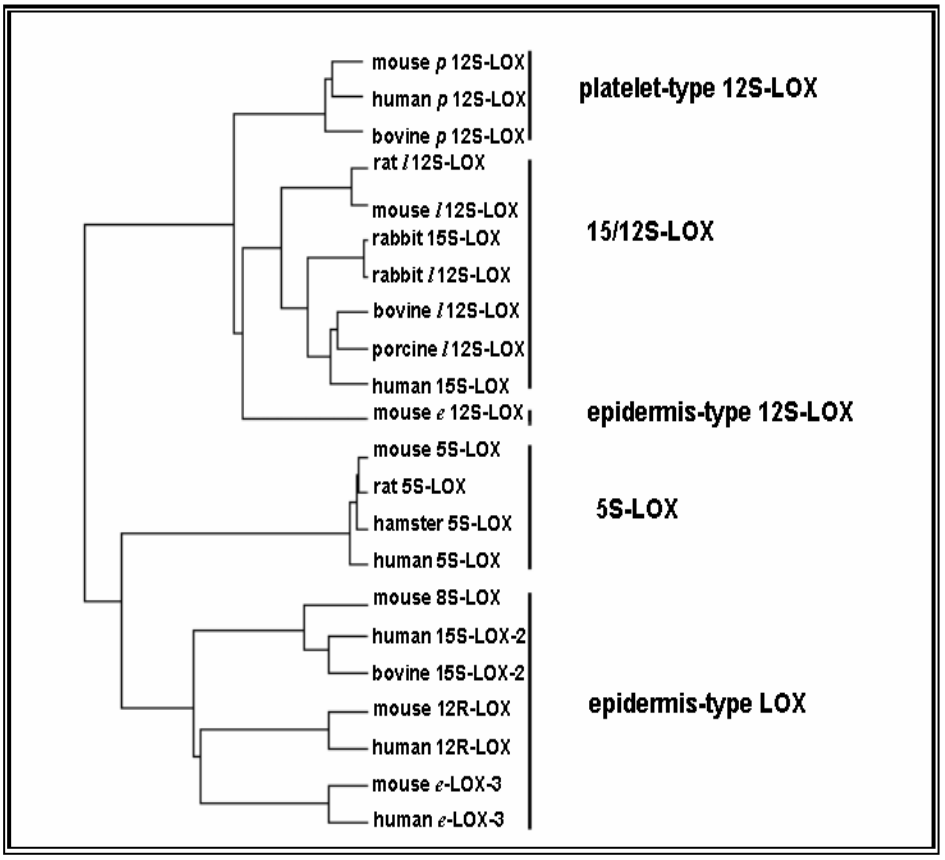


Fig 7. Phylogenetic tree of mammalian lipoxygenases. (Fürstenberger et al., 2002).

In humans eight distinct lipoxygenase genes have been identified; six of them are functional (ALOX5, ALOX12, ALOX15, ALOX15B, ALOX12B, ALOXE3) and two are pseudogenes (ALOX12P1, ALOX12P2) (Sun et al., 1998). In mice, the total number of lipoxygenase genes has now reached seven Alox5, Alox12p, Alox12l, Alox12e, Alox8, Alox12b and Aloxe3 (Kinzig et al., 1999). Based on the phylogenetic relatedness (Fig. 7) mammalian LOX is classified as i) 5-LOX, ii) platelet-type 12-LOX, iii) 15/12-LOX (reticulocyte-type 15-LOX-1 and leukocyte-type 12-LOX, both exhibiting a dual positional

specificity) and iv) epidermis-type LOX (12R-LOX, 15-LOX-2, 8-LOX, epidermis-type LOX-3).

1.3.4. Structural Aspects:

Although LOXs have been investigated for more than 50 years, their crystal structure was only solved recently. The first complete set of X-ray coordinates became available for the soybean LOX-1 (Boyington et al., 1993). Later on, the data were refined to 1.4Å resolution (Minor et al., 1996). More recently, the crystal structure of the soybean LOX-3 was reported (Skrzypczak-Jankun et al., 1997) and now X-ray coordinates for LOX-3/ligand complexes are available (Pham et al., 1998). The first crystallization of a mammalian LOX (rabbit reticulocyte-type 15-LOX) was described in 1990 (Sloane, 1990) but it took about 7 years to solve the crystal structure (Gillmor, 1997). Although the data set is incomplete, it gives a good impression on the structural features of mammalian LOXs. All LOXs have a two-domain structure. The small N-terminal domain (eight-stranded anti-parallel β-barrel, amino acids 1–115) is similar in sequence, size, and structure to an analogous C-terminal-barrel domain in mammalian lipases. It may play a role in localizing the enzyme near substrates, which may be sequestered in biomembranes or lipoproteins. The large C-terminal catalytic domain shares a 1600 Å interface with the N-terminal β-barrel and both domains are loosely associated with each other. It contains the catalytic non-heme iron and consists of 18 helices that are interrupted once by a small ß-sheet sub-domain. The core of the catalytic domain contains two long central helices, one of which adopts a α-helix conformation for the length of several residues (360–366 and 537–543). This α-helical section contains four of the five protein iron ligands (H361, H366, H541, H545). The fifth ligand constitutes the C-terminal I663. The crystal structure of the soybean LOX-1 indicated a water molecule as sixth iron ligand (Minor et al., 1996). This ligand position, which is positioned *trans* to H691 (aligns with H541 of the rabbit enzyme), is located at the

boundary of a large internal cavity and may be displaced by substrate fatty acids or competitive inhibitors. These ligands coordinate the iron atom with an excellent octahedral geometry. X-ray absorption studies are supportive for an octahedral iron ligand sphere but suggested a considerable degree of distortion (Solomon et al., 1997 and Kuban et al., 1998). In the first report of the 3D-structure of the soybean LOX-1 two cavities in the major domain were described (Boyington et al., 1993). Cavity I, which was suggested as path for

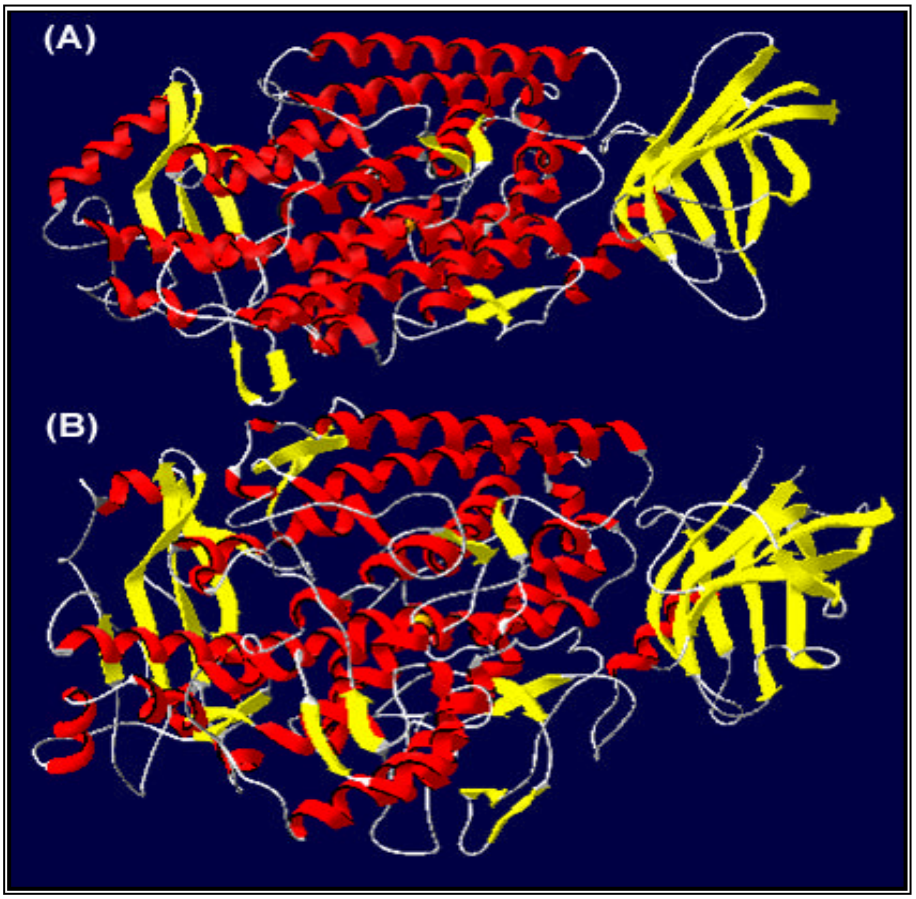


Fig. 8. Two-domain structures of animal and plant lipoxygenases. (A) Rabbit reticulocyte 15S-lipoxygenase. (B) Soybean LOX-1 structure. α-helices are shown in red and β sheets are yellow in color.

the movement of dioxygen, forms an 18 Å long hydrophobic tunnel. The second cavity (fatty acid binding site) is 40Å long and is lined by the side chains of 46 mainly hydrophobic amino acids, most of which are highly conserved among LOXs. This cavity has two major bends. One is adjacent to

the non-heme iron close to the bottom of the cavity, and the second bend is at the middle of the cavity, where it becomes very narrow. In the 1.4Å structure, cavity I (oxygen path) is less obvious. In fact, the 1.4Å model does not allow transport of dioxygen because of steric constrains. In the structure of the rabbit 15-LOX, there is no clear evidence for a second cavity, which may serve as oxygen access channel to the active site. Thus, it was concluded that the rate of oxygen insertion is diffusion controlled and that the enzyme does not specifically position the oxygen molecule relative to the substrate fatty acid. Consequently, the stereospecificity of the LOX reaction may be determined by the forced conformation of the substrate and/or by stabilizing a certain structure of the transition state.

1.3.5 Substrate alignment and Positional specificity:

The substrate-binding cleft of mammalian 15-LOXs is a boot-shaped cavity, which is directly accessible from the surface of the protein (Gillmor et al., 1997). Experiments with a set of arachidonic acid isomers, in which the double bond system is shifted gradually towards the methyl or carboxyl terminus of the fatty acid, suggested that the oxygenation rate and the positional specificity of 15-LOXs depend on the distance of the doubly allylic methylene from the methyl end of the substrate (Kühn et al., 1990). From these data, it was concluded that for the reticulocyte-type 15-LOX substrate fatty acids may slide into the active site with the methyl end ahead. Mutagenesis experiments and modeling of enzyme–substrate complexes (Gan et al., 1996) suggested a two-point enzyme/ substrate interaction: (i) the positively charged R403, which is localized at the entry of the substrate binding cleft may interact with the negatively charged fatty acid carboxylate, (ii) the amino acids F353 (Borngräber et al., 1996), I418, and I593 (Sloane et al., 1991 and Borngräber et al., 1999) of the rabbit enzyme form the bottom of the boot-shaped substrate binding cleft and thus, appear to interact with the methyl terminus of the substrate (Gillmor et al., 1997). Mutagenesis of R403

of the human 15-LOX to a neutral leucine strongly impaired both, substrate affinity and the reaction rate (Gan et al., 1996). The human 5-LOX does also contain a positively charged amino acid at this position (K409). However, K409L mutation does not influence the reaction rate and thus, this residue may not be important for substrate alignment at the active site of this LOX-isoform. It remains to be seen whether the substrate carboxylate may play a role in substrate alignment at the active site of 5-LOXs.

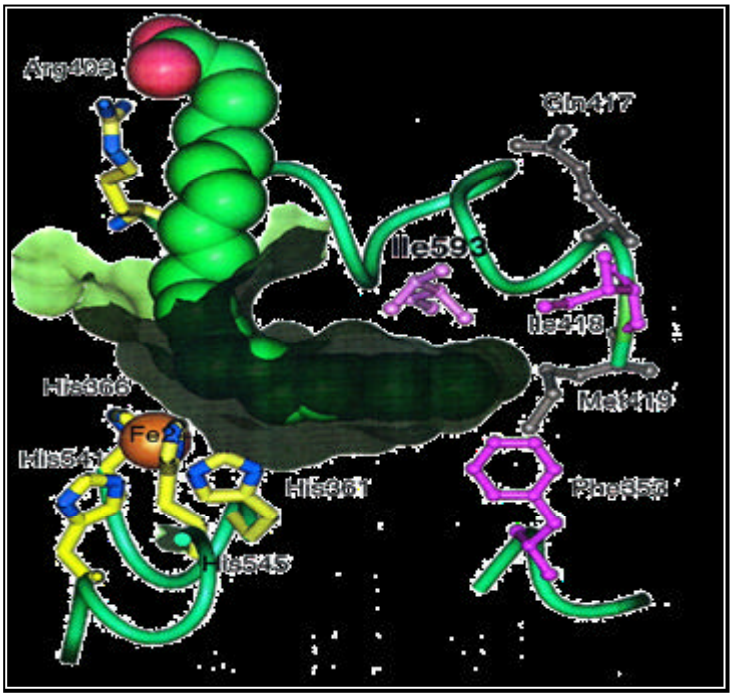


Fig. 9. The positional determinants of rabbit reticulocyte 15-LOX. The positional determinants are indicated in violet with the catalytically active iron and docked in arachidonic acid. (Borngräber et al., 1999).

To rationalize the mechanistic differences between arachidonic acid 5- and 15-lipoxygenation two alternative hypotheses have been put forward (Funk and Loll, 1997): (i) the "orientation-based" hypothesis suggests that for 15-lipoxygenation, arachidonic acid may slide into the substrate binding pocket with its methyl terminus ahead and may adopt a steric configuration at the active site favoring oxygen insertion at C-15 of the arachidonic acid backbone. In contrast, for arachidonic acid 5-lipoxygenation an inverse, head-

to-tail substrate orientation was assumed (Prigge et al., 1998). (ii) According to the "space-based" hypothesis, the substrate alignment at the active site is conserved among all LOX isoforms and the volume of the substrate-binding pocket appears to be decisive for the positional specificity (Browner et al., 1998). In fact, the substrate-binding cavity of the human 5-LOX is about 20% bigger than that of the rabbit reticulocyte-type 15-LOX (Gillmor et al., 1997; Browner et al., 1998) and the additional space may allow an optimal substrate orientation for 5-lipoxygenation.

To test the orientation-based hypothesis, a combined strategy of targeted substrate modification and site directed mutagenesis was used. It is known for quite a while that 15-H(p)ETE is oxygenated by the soybean LOX-1 to 5*S*, 15*S*-, and 8*S*, 15S-DiHpETE. Thus, the enzyme is capable of catalyzing 5*S*-lipoxygenation of 15*S*-HpETE. When the substrate's carboxylate was methylated, the share of 5-lipoxygenation was strongly increased. In fact, 5-lipoxygenation was the major reaction (85%) catalyzed by both the soybean and the rabbit 15-LOX using 15-HETE methyl ester as substrate (Schwarz et al., 1998). When I418 of the rabbit enzyme was mutated to a less space-filling A (deepening of the substrate-binding pocket), a large share (90%) of 8-lipoxygenation was observed. These data suggest that 15-HETE methyl ester slides into the active site with its carboxylate ahead (inverse substrate orientation) approaching C-7 to the enzyme's hydrogen acceptor, which is a prerequisite for 5-lipoxygenation. If the substrate-binding pocket is deepened (I418A), the substrate may slide in farther to approach C-10 to the hydrogen acceptor leading to an increased share of 8-lipoxygenation products (Schwarz et al., 1998). Although these data support the "orientation hypothesis" as mechanistic basis for 15-LOX-catalyzed 5-lipoxygenation, they do not exclude the possibility that the space hypothesis may be applicable for arachidonic acid 5-lipoxygenation catalyzed by the human 5-LOX. In fact, there is kinetic evidence suggesting that 5- and 15-LOX-catalyzed 5-lipoxygenation may be mechanistically distinct. 15-LOX-catalyzed 5-

lipoxygenation is strongly stimulated by methylation of the substrate's carboxylate. In contrast, the human 5-LOX does not accept methyl arachidonate as substrate.

To test whether or not the "space-related hypothesis" may be applicable for the human 5-LOX site directed mutagenesis studies were carried out. In these experiments the small sequence determinants for the positional specificity were mutated to the larger counterparts present in the rabbit 15-LOX. This strategy reduced the volume of the substrate-binding pocket. It was found that multiple site-directed mutagenesis gradually altered the positional specificity of the human 5-LOX from 5*S*- via 8*S*- to 15*S*-lipoxygenation (Schwarz et al., 2001). This data clearly indicate that space may be an important parameter for the positional specificity of the human 5-LOX.

1.3.6. R-Lipoxygenases:

The term *R*-LOX arose in the 1980s following the unexpected discovery of LOX enzymes in marine invertebrates that converted arachidonic acid to hydroperoxyeicosatetraenoic acids (HPETEs) with the *R* stereoconfiguration (Bundy et al., 1986 and Hawkins et al., 1987). This finding was in distinct contrast to almost all that was known about LOX transformations in animals and plants where the biosynthesis of *S*-configuration products was the rule. It was, therefore, not certain whether the *S*-LOX and *R*-LOX were members of the same enzyme family. For example, the structurally unrelated hemoprotein, cyclooxygenase (COX), also oxidizes polyunsaturated fatty acids to fatty acid peroxides (Hemler et al., 1978).

Initially, mechanistic studies supported the hypothesis that S- and R-lipoxygenase belong to the same enzyme family. As S-LOXs, *R*-LOXs convert polyunsaturated substrates to fatty acid hydroperoxides (Hawkins et al., 1989).

Both enzyme types initiate catalysis by the stereoselective hydrogen removal from a bisallylic methylene and both exhibit an "antarafacial relationship" between hydrogen abstraction and oxygen insertion (i.e. the two processes occur on opposite faces of the substrate molecule). Purification and molecular cloning of an 8*R*-LOX from coral eventually established the relatedness of the "two classes" of LOX enzyme (Brash et al., 1996). We now consider the two LOX-types as members of the same enzyme family, which exhibit opposite stereo-control of the oxygenation reaction.

1.3.6.1. R-LOXs in aquatic invertebrates

Marine invertebrates were the first organisms in which *R*-LOX activities were discovered. Bundy et al. originally described an arachidonate 8*R*-LOX activity in the coral *Pseudoplexaura porosa* (Bundy et al., 1986). Subsequently, 8*R*-LOX has been found in all other species of coral examined, with geographical distributions ranging from the Caribbean to Japan ɒ the Arctic (Brash et al., 1987; Corey et al., 1987; Varvas et al., 1999). Other marine sources of *R*-LOX activities now include 8*R* in starfish (Meijer et al., 1986), barnacle (Hill et all., 1992), crab (Hampson et al., 1992) and sea squirt (Knight et al., 1999), 5*R* and 8*R* in surf clam (Hada et al., 1997), 8*R* and also 12*S* in *Aplysia californica* (Steel et al., 1997), 11*R* and 12*R* in sea urchin (Hawkins et al., 1989) and 11*R* and also 12*S* in mussel (Coffa et al., 2000). Freshwater and marine species of *Hydra* synthesize 11*R*-HETE and 12*S*-HETE (Di Marzo et al., 1994).

1.3.6.2. Bioactivities of invertebrate R-hydroperoxides

The 8*R*-LOX activity in certain species of starfish is involved in the reinitiation of meiosis in oocytes (oocyte maturation) (Meijer et al., 1986). Starfish oocytes develop in the ovary to a stage where they remain arrested in the first prophase of meiosis. Upon spawning, the oocytes receive an

extracellular chemical signal from the surrounding follicle cells to reinitiate meiosis, leading to their transformation to mature eggs that are susceptible to fertilization. In starfish, a universal chemical signal for maturation is 1-methyladenine, but in about half of all species tested, 8*R*-HETE is also active and fully competent as a maturation hormone (Meijer et al., 1986). The 8*R*-HETE is active on the outside of the oocyte and there is circumstantial evidence for the existence of a G-protein-coupled 8*R*-HETE receptor (Hughes et al., 1994). Other notable examples of the bioactivity of *R*-configuration HETEs in aquatic invertebrates include the involvement of 11*R*-HETE in tentacle regeneration in *Hydra* (De Petrocellis and Di Marzo, 1994) and 8*R*-HETE as a hatching factor in barnacles (Hill et al., 1992).

1.3.6.3. Fungal and algal R-LOX

The "take all" fungus of wheat *Gaeumannomyces graminis*, secretes a LOX into the growth medium. This highly glycosylated LOX has been purified, micro sequenced and cloned (Su et al., 1998); the primary sequence is clearly related to other LOX. The catalytic metal has been shown by atomic emission spectroscopy to be manganese rather than iron (Su et al., 1998). The enzyme also displays unusual catalytic activity. Linoleic acid is the best natural substrate, being converted to the bis-allylic 11*S*-hydroperoxide and the more conventional LOX structure, albeit of *R* configuration, 13*R*-HPOD (Hamberg et al., 1998). Analysis of eicosanoid metabolites in the temperate red alga *Rhodymenia pertusa* has provided strong circumstantial evidence that this alga contains a 5*R*-LOX acting on both arachidonic and eicosapentaenoic acids (Jiang et al., 2000).

1.3.6.4. R-Oxygenation in higher plants

To date no *R*-LOX has been described in higher plants. Formation of *R*-configuration hydroperoxides is invariably accompanied by the formation of

S-hydroperoxides as well. The enzymes shown to produce *R*-configuration products are the so-called type-II LOX enzymes from seeds of leguminosae like soybeans and peas. At their slightly acidic pH-optimum, these LOX-isoforms form racemic mixtures of 9- and 13-HODE (Van Os et al., 1979) and they appear to function as catalysts of stereo-random fatty acid oxygenation. When the pH of the incubation reaction was raised to pH 9.0, the type-II LOX from soybeans and peas formed 9-HODE as major product in 2:1 ratio with 13-HODE. In the case of the soybean L-2 isozyme, the 9-HODE product was 78% 9*R* and the 13-HODE product was 91% 13*S* (Van Os et al., 1979). Thus, at elevated pH the type-II LOXs appear to gain more control over the oxygenation reaction to form more specific reaction products.

1.3.6.5.Human 12R-LOX

One of the six functional human LOX genes, the *ALOX12B* gene, encodes a 12*R*-LOX, and the enzyme forms 12*R*-HpETE from arachidonic acid with high specificity (Boeglin et al., 1998).

The *ALOX12B* gene is located on chromosome 17p13.1, where it forms a cluster of epidermis-type LOX together with two other recently discovered LOXs, the second type of 15*S*-LOX (15-LOX-2) and eLOX-3, an enzyme which encodes a hydroperoxide isomerase and was implicated in skin differentiation (Yu et al., 2003). Human 12*R*-LOX has very limited tissue distribution. To date, only human normal and psoriatic skin and tonsils have been found to express the enzyme and to convert exogenous arachidonic acid to 12*R*-HETE. The levels of 12*R*-LOX and its oxygenation products appear to be elevated in psoriasis and other proliferative skin dermatoses (Baer et al., 1991; Baer et al., 1995). In tonsils, the enzyme is expressed in the stratified squamous epithelium, and is absent from the underlying lymphatic tissue including the germinal centers as determined by *in situ* hybridization (Schneider et al., 2001). Using RT-PCR techniques, Krieg et al. (2001)

detected expression of RNA in additional tissues like lung, testis, adrenal gland, ovary, prostate, and skin; lower abundance was detected in salivary and thyroid glands, pancreas, brain, and plasma blood leukocytes. The intron/exon boundaries in all human LOX genes are highly conserved. The *ALOX12B* gene differs from other LOX in its gene structure by having an additional intronic sequence that divides exon 4. As a result, the *ALOX12B* gene is split into 15 exons and 14 introns and spans about 15 kb. The closely related human *ALOXE3* gene also contains the additional intron 4b (Krieg et al., 2001). A structural characteristic of the human 12*R*-LOX is the presence of a proline-rich 31 amino acid insert within exon 4 between amino acids Leu150 and Pro182 (or Pro154 and Ile186, depending on the alignment) (Boeglin et al., 1998). Assuming an overall similar three-dimensional structure of all LOXs, it is conceivable that this stretch of amino acids forms a loop on the surface of the protein located after the first helix of the globular catalytic domain (Schneider et al., 2002). BLAST searches revealed no significant homology of this amino acid stretch to any other protein except for the mouse homologue. It might be hypothesized that this amino acid insert, located on the surface of the protein, is involved in subcellular localization of the enzyme or interaction with other proteins.

1.3.6.6. Mouse 12R-LOX

The orthologue of the human *ALOX12B* gene in the mouse (*Alox12b*) also encodes a 12*R*-LOX (Krieg et al., 1999). The mouse *Alox12b* gene and protein share the structural features typical of the human 12*R*-LOX, i.e. the additional intron placed in exon 4, and the additional proline-rich 31 amino acid insert at the beginning of the catalytic domain of the protein. Mice have a total of seven functional LOX genes, one more than humans, and all encode *S*-LOX except for the *Alox12b* and the *Aloxe3* genes (Kinzig et al., 1999). The additional seventh gene encodes an epidermis-type 12*S*-LOX; the corresponding gene in humans is a pseudogene (Sun et al., 1998). Mouse 12*R*-

LOX mRNA is strongly expressed in stratifying epithelia in adult animals. Using RT-PCR methods, highest levels were detected in back skin, foot-sole epidermis, forestomach, trachea, and tongue; lower levels were found in lung, liver, kidney (Heidt et al., 2000) and cerebral cortex (Sun et al., 1998). The expression during embryonic development is highly regulated with mRNA being not detected up to day E14.5, but with onset of expression at day E15.5 and intense expression at day E16.5 throughout the epidermis, in the nasal epithelium, and on the upper surface of the tongue. A role for 12*R*-LOX in embryonal skin development has been implicated (Sun et al., 1998). Different from the human 12*R*-LOX, enzymatic activity of the mouse 12*R*-LOX has not been detected in incubations of tissue samples with free exogenous arachidonic acid. The reason for this unexpected result is that the murine enzyme only reacts with arachidonic acid methyl esters and only exhibits a minor activity with free arachidonic acid (Krieg et al., 1999; Siebert et al., 2001). Since fatty acid methyl esters are not abundant in mammalian cells the native substrate of the murine 12R-LOX is still unknown.

1.3.6.7. Mechanistic aspects of R-oxygenation

R-LOXs do not constitute a separate branch within the LOX superfamily, a structural motif that would signify either an *R*-LOX primary sequence or an *R*-oxygenation active site has not been found. The high degree of sequence conservation between the human 12*R*-LOX and the 15*S*-LOX-2 (˜50% amino acid identity) argues for a similar architecture of the active sites. Unfortunately, the availability of X-ray structures of several LOXs has not afforded detailed insights into the substrate alignment at the active site. Unfortunately, no crystal structure of a LOX-substrate complex is available to date. However, even in the presence of such data conclusions on the productive enzyme/substrate complexes would be rather difficult since there is a high probability for substrate movement within the active site. Currently,

there is much more experimental data on how LOX control the regio-selectivity of oxygenation reaction when compared with the stereo-specificity.

OBJECTIVES OF THE STUDY

Most of the mechanistic studies related to the structural basis of the positional specificity of mammalian LOXs have been carried out on the classical S-LOXs, such as the rabbit and human 15-LOX-1, the leukocyte-type 12-LOX and the human 5-LOX. Unfortunately little is known about the stereochemical control mechanisms of the recently discovered R-LOXs. To investigate the structural basis for the positional and stereochemical control mechanisms of mammalian R-LOX in more detail we have undertaken the present study and applied an integrated research strategy involving targeted substrate modification and site-directed mutagenesis. The specific objectives of the present study are:

- To investigate the primary determinants involved in the positional specificity of the murine 12R-LOX.

- To find the amino acid determinants involved in the stereospecificity of the enzyme.

- To investigate the determinants involved in different substrate specificities of mouse and human 12R-LOX.

- To study the importance of the putative fifth iron ligand, Asn582 in the enzyme catalysis

- To investigate the importance of the proline-rich extra stretch of amino-acids unique to the mammalian R-lipoxygenases.

MATERIALS AND METHODS

2.1. CHEMICALS AND BIOCHEMICALS:

The following biochemicals were used in this study

Arachidonic acid *(cis, 5, 8, 11, 14-Eicosatetraenoic acid)* and its methyl ester Linoleic acid *(cis, 9, 12-octadecadienoic acid)* and its methyl ester α-linolenic acid *(cis, 9, 12, 15-octadecatrienoic acid)*	Sigma, Deisenhofen, Germany
HPLC standards: 5-, 8-, 9-, 11-, 12-, 15-HETEs HPLC solvents: Methanol, n-Hexane, Isopropanol, Acetic acid (HPLC grade)	- Cayman Chemicals, USA - Baker (Deventer, Netherlands)
Antibodies: RGS-His (mouse) Anti mouse-IgG peroxidase conjugate	- Qiagen, Hilden, Germany - Sigma, Deisenhofen, Germany
Nickelagarose (Ni-Agarose) Imidazol	- Qiagen, Hilden, Germany - Serva, Heidelberg, Germany
Bacto-Agar, Bacto-Trypton, Yeast Extract Ampicillin (sodium salt), IPTG (Dioxanfree) Kanamycin	- Difco, Detroit, USA - Roth, Karslruhe, Germany - Sigma, Deisenhofen, Germany
Acrylamide/Bisacrylamide TEMED Tris	- Roth, Karlsruhe, Germany - Serva, Heidelberg, Germany - Merck, Darmstadt, Germany
Agarose Ethidium bromide	- Promega, Mannheim - Roth, Karlsruhe, Germany
Sf9 insect cells, TNM-FH media Insect express Sf9-S2 serum free medium Fetal Calf Serum Penicillin and Streptomycin	- BD Biosciences Pharmingen, USA - PAA laboratories, - Gibco™, Invitrogen, USA - Gibco™, Invitrogen, USA

All the other chemicals not mentioned above were from Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (Deisenhofen), Germany.

2.1.1. Enzymes and Kits:

Alkaline Phosphatase	Roche, Mannheim, Germany
DNA Ligase, Bacteriophage T4	Roche, Mannheim, Germany
Restriction endonucleases	New England Biolabs, Schwalbach
BD Advantage ™ 2 Polymerase Mix	BD Biosciences Clontech, San Diego (USA)
DNA Polymerase, *Thermus aquaticus* (Taq)	Promega, Mannheim, Germany
Pfu turbo DNA polymerase	Stratagene, La Jolla (USA)
M-MLV Reverse transcriptase	Promega, Mannheim, Germany
BaculoGold™ Transfection Kit	BD Biosciences Pharmingen, San Diego,(USA)
Miniprep Spin and Plasmid-Midi-Kit	Qiagen, Hilden, Germany
Qia quick Gel extractions kit	Qiagen, Hilden, Germany
QuikChange Site-Directed Mutagenesis Kit	Stratagene, La Jolla (USA)
Rneasy Mini ki	Qiagen, Hilden, Germany
Western Lightning Chemiluminescence plus	Perkin Elmer, Boston (USA)

2.1.2. Plasmids and *E.Coli* Host Strains:

Plasmid Vectors:

PCR II-TOPO	Invitrogen, USA
pQE-80L	Qiagen, Hilden, Germany
pVL 1392	BD Biosciences Pharmingen, USA
pWhitescript	Stratagene, La Jolla (USA)

***E. coli* host strains:**

M15[prep4]	Qiagen, Hilden
Top10	Invitrogen, Karlsruhe
XL-1 Blue	Stratagene, La Jolla (USA)

The murine 12R-LOX cDNA cloned into the pQE-80L was a gift from Prof. Fürstenburger group, Heidelberg, Germany and was used for expression of protein in bacterial cells. The murine 12R-LOX cDNA was cloned into pVL 1392 transfer vector between EcoRI and XbaI restriction sites and is used for expression in insect cells.

2.1.3. Primers:

The following primers are designed for the site-directed mutagenesis studies in murine 12R-lipoxygenase. The primers were synthesised by BioTez Berlin-Buch GmbH, Berlin and TIB MOLBIOL, Berlin, Germany.

1. PhenylalanineF390 (*Borngräber I determinant*) Mutational studies:

1. F390L	CTA CGC GGA G**TT G**TA CAG CCA CGA
2. F390A	CTA CGC GGA G**GC C**TA CAG CCA CGA

2. Alanine455 (*Sloane Determinant*) Mutational studies:

1. A455I	CTC TCT GCC AGG **ATC** ATG TCC CTG
2. A455F	CTC TCT GCC AGG **TTC** ATG TCC CTG
3. A455W	CTC TCT GCC AGG **TGG** ATG TCC CTG

3. Valine631 (*Borngräber II determinant*) Mutational studies:

1. V631I	TGT GCT CTT G**AT A**CT CTG GAC TCT
2. V631F	TGT GCT CTT G**TT T**CT CTG GAC TCT
3. V631W	TGT GCT CTT G**TG G**CT CTG GAC TCT
4. V631A	CAT TGT GCT CTT G**GC G**CT CTG GAC TCT
5. V631G	CAT TGT GCT CTT G**GG G**CT CTG GAC TCT

4. Glycine441 (*Brash determinant*) Mutational studies:

1. G441A	AAT AGC ATT **GCG** CGG GCC CT
2. G441V	AAT AGC ATT **GTG** CGG GCC CT

5. Iron ligands:

1. N582S	AGG CAT GCA GCT GTC **AGC** TCA GGC CAG CTG GAG
2. N582H	AGG CAT GCA GCT GTC **CAC** TCA GGC CAG CTG GAG

6. Deletion studies:

1. 154P185P	AAG GAC TTC TAC CAC TGG AGG GTC TTT GTT ATC AAC ATT AAG GCT ACC AGG TTC TTA AAC
2. 154P159I	AAG GAC TTC TAC CAC TGG GTC TTT GTT TAT GTG GAT ATC CCC AGT TAC CAT CCT CCT
3. 180Y185P	CCC AAC CGG CCT GAG TGG GAT GGT TAT ATT ATC AAC ATT AAG GCT ACC AGG TTC TTA AAC

7. Primers for reverse transcription of human 12R-lipoxygenase from human HaCaT cells:

1. h12RT up	CTT GCC TGG CCT TCC TGA GGA ACC TG
2. h12RT dw	GTT GAC AGC AGC ATG CTT GGC AGA GC

2.2. MOLECULAR BIOLOGY TECHNIQUES:

For the protocols of the standard molecular biology techniques, the cloning manual "Molecular Cloning: A laboratory Manual" (Sambrock et al., 1989) was followed. The molecular biology techniques used were restriction digestion of plasmid DNA using restriction endonucleases, ligation of the restriction fragments by DNA ligases, agarose electrophoresis, gel extraction purification of restriction fragments, transformations into competent *E.coli* cells, DNA plasmid extraction and purifications etc.,

2.2.1. Expression of murine 12(R)-LOX in bacteria and its purification on Ni-NTA metal-affinity column:

2.2.1.1. Construction of the recombinant vector plasmid:

The murine 12R-LOX cDNA was cloned into the pQE-80L plasmid with the 6xHis-affinity tag placed in front of the N-terminus of the protein. The recombinant plasmid was expressed as His-tag fusion protein, which enabled efficient purification on Ni-NTA column. The 12R-LOX cDNA was inserted between BamHI and SalI restriction sites in the polylinker site of the pQE-80L vector.

The recombinant plasmid was transformed into M15 (pREP4) bacterial cells and plated on LB-agar plates with kanamycin and ampicillin. The correct recombinants were selected by restriction digestion of the isolated plasmid DNA of different bacterial colonies. The selected clones were first tested for expression in small-scale cultures and later on in large-scale fermentation samples.

2.2.1.2. Expression of the recombinant protein:

The selected recombinant 12R-LOX clones were grown in 1 litre LB medium containing both ampicillin (100µg/ml) and kanamycin (25µg/ml). It was grown at 37° C with shaking until the OD at 600 nm was 0.5-0.7. Adding IPTG to a final concentration of 1 mM induced the protein expression. The culture was grown additionally for two more hours and then the bacterial pellet was harvested. The cells were lysed in PBS with the French press (Emulsiflex C5, Avestin) at 20,000 psi and the lysate was centrifuged in centrifuge (SS34 Rotor) at 3000 rpm for 30 minutes at 4° C. Aliquots were taken at each step for monitoring the enzyme activity.

2.2.1.3. Purification:

Purification of the recombinant protein was performed using nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography. 50% Ni-NTA slurry (500 µl for 1 l culture) was added to the cleared bacterial lysate and mixed gently by shaking at 4° C for 60 min. Then the lysate- Ni-NTA slurry was loaded into a column and washed twice with wash buffer containing 20 mM imidazole. The imidazole ring is a part of the structure of histidine so imidazole itself can also bind to the nickel ions. At low imidazole concentrations, non-specific, low affinity binding of background proteins is prevented. In contrast, the 6xHis-tagged protein binds strongly to the Ni-NTA matrix and thus is not removed under these conditions. After washing the column to remove unspecific proteins, the 12R-LOX protein was eluted with 250 mM imidazole, which displaces the His-tag fusion protein from the matrix. The purified protein was fractionated and the fractions were analysed further by SDS-PAGE and Western blotting analysis.

2.2.2. Expression of murine 12(R)-LOX in insect cells and its purification:

2.2.2.1. Construction of pVL 1392-12(R)-LOX:

The murine 12R-lipoxygenase cDNA was transfected and expressed in Sf9 insect cells using BaculoGold^{TM} Transfection Kit. The Baculovirus Expression Vector System (BEVS) is a versatile eukaryotic expression system and is preferred over the bacterial system for various reasons. A post-translational modification is possible in the insect cells and high-level expression of a cloned gene can be achieved. BaculoGold^{TM} DNA is a modified AcNPV baculovirus DNA that contains a lethal deletion and does not code for viable virus. Co-transfection of the BaculoGold^{TM} DNA with a complementing baculovirus transfer vector rescues the lethal deletion by homologous recombination. The pVL1392 Baculovirus transfer vector

contains the complete polyhedron gene locus but lack part of the polyhedron gene-coding region. Homologous recombination occurs during co-transfection of the pVL 1392 transfer vector with gene of interest and BaculoGold^{TM} DNA. The murine 12R-LOX cDNA including the his-tag was inserted into the pVL 1392 vector between EcoRI and XbaI restriction sites in the multiple cloning site of the vector using the conventional cloning protocols. The recombinant DNA was transformed in to TOP 10 competent bacterial cells and plated on LB-Agar plates containing 100µg/ml Ampicillin. The correct recombinant clones were selected by restriction digestion analysis and by sequencing using the pVL 1392 vector sequencing primers.

2.2.2.2. Transfection of the recombinant transfer vector in to Sf9 insect cells:

5 µg of highly purified recombinant baculovirus transfer vector containing 12R-LOX (pVL-12R-LOX) was used for transfection procedure. It was mixed with 1 µg BaculoGold^{TM} DNA and gently vortexed and left for 5 min before adding 1 ml Transfection buffer B (25 mM Hepes, pH 7.1; 125 mM $CaCl_2$; 140 mM NaCl). Sf9 cells ($2x10^6$ cells / plate) in TNM-F medium were seeded onto a 60 mm tissue culture plate (Falcon) and left for 5 min for the attachment of the cells to the plate. After the cells were attached and about 50-70% confluent, the old medium was aspirated and replaced with 1 ml of Transfection buffer A (Grace's Medium supplemented with 10% FBS). The 1 ml of Transfection Buffer B/DNA solution was added drop-by-drop to the Sf9 insect cell culture plate and rocked gently. A fine, white milky colour calcium phosphate/DNA precipitate was formed. The plate was incubated at 27° C for 4 h. After 4 h, the transfection medium was replaced with fresh TNM-FH medium and incubated again for 3 days at 27° C. The transfected cells show signs of infection where cells appear larger than the uninfected ones, have enlarged nuclei, stop dividing and often float in the medium. For the positive control pVL1392-XylE Control Transfer Vector DNA was used with the

BaculoGold™ DNA. The insect cells transfected with this recombinant DNA express XylE protein, which turns the transfected cells bright yellow when 100 µl catechol solution is added to the cells. Transfection supernatant was amplified by reinfection to fresh Sf9 cells to produce high titer virus stocks. To determine viral titer, in plaque-forming units per ml (pfu/ml), the plaque assay was made. With this assay the amount of virus supernantant required to infect the cells was determined.

2.2.2.3. Plaque assay:

2 x 10^7 cells in TNM-FH insect medium were plated per well on a 6 well plate (Falcon) and the cells were allowed to attach firmly to the plate. Serial dilutions were made with the virus transfection supernatants (10^{-4}, 10^{-5}, 10^{-6}) and 100 µl of each dilution was added to each well of the plate. The plate was gently rocked to spread the virus supernatant and then incubated for 1 hour at 27° C to allow the virus particles to infect the cells. The dilutions and infection was done in duplicates. 2 % Agarplaque-Plus Agarose (BD Biosciences Pharmingen) solution was prepared in protein free medium. (1 g in 50 ml protein free medium) and cooled to 45° C in water bath. Equal volumes of Agarose solution and pre-warmed TNM-FH medium were mixed and the temperature of the mixture was maintained at 37° C. The virus supernatant from the wells was removed and overlaid with 3 ml of molten overlay mix to each well. The plates were allowed to dry a bit and solidify and then incubated for 6-10 days. To visualise the plaques 1ml of 0.5 % Neutral Red solution was added to each well, incubated overnight and the plaques counted.

2.2.2.4. Calculating the titer:

The virus titer was calculated by the following formula:

pfu/ml = 10 (# infected cells / foci) (dilution factor).

For calculating MOI (multiplicity of infection) the following formula was used (please use formel editor of the Microsoft program):

$$\text{Inoculum required (ml)} = \frac{\text{Desired MOI (pfu/cell)} \times \text{(total No. of cells)}}{\text{Titer of viral inoculum (pfu/ml)}}$$

2.2.2.5. Expressing recombinant proteins in Sf9 cells for protein purification:

Recommended maximum infection densities for the production of recombinant protein in a monolayer culture with serum free medium is 0.5 – 1 x 10^6 cells / ml. 1 x 10^6 cell / ml in 75 cm (Falcon) culture plate was plated and when the cells attached to the bottom, the virus supernatant with MOI of 5 was added to the monolayer and incubated for 3 days at 27° C. 3 days after infection when the cells are enlarged in size (about 2-fold) and a large nucleus was visible the cells were harvested and the cells were spun down at 3000 rpm for 15 minutes. The cells were washed once with cold PBS and suspended in 3 ml of PBS. The cells were lysed by sonication on ice with six 15 sec bursts at 75 W with 10 s cooling period. The lysate was centrifuged at 14000 rpm for 10 minutes. The purification protocol is the same that was followed for the bacteria expressed protein purification using nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography.

2.2.3. Site-directed Mutagenesis of murine 12(R)-LOX:

Mutations in murine 12R-lipoxygenase (pQE-12R-LOX and pVL-12R-LOX) were performed using the Stratagene`s QuikChange site-directed mutagenesis kit. This is a rapid procedure, which requires no specialised vectors, unique restriction sites or multiple transformations and generates mutants with greater than 80% efficiency. The kit was used to make point mutations and to delete multiple amino acids in murine 12R-lipoxygenase.

The procedure requires supercoiled double-stranded DNA (dsDNA) vector with the insert of interest (pQE-80L-12R-LOX and pVL-12R-LOX) and two synthetic oligonucleotide primers containing desired mutation (see 2.1.3). The oligonucleotide primers, each complementary to opposite strands of the vector, were extended during polymerase chain reaction by Pfu Turbo DNA polymerase. Incorporation of the oligonucleotide primers generates mutated plasmid containing required mutation. After the polymerase reaction, the product was treated with DpnI endonuclease. The DpnI endonuclease (target sequence: 5´-Gm^{6}ATC-3´) is specific for methylated and hemi methylated DNA and was used to digest the parental DNA template and to select for mutation containing synthesised DNA. DNA isolated from almost all *E.coli* strains is dam methylated and therefore susceptible to DpnI digestion. The nicked vector DNA containing the desired mutation was transformed into XL-1 Blue super-competent cells. The cells were plated on LB-Agar plates with ampicillin (100µg/ml). The clones with correct mutations were selected with restriction digestion analysis and by sequencing the mutated plasmid DNA.

PCR Mixture:

1. 5 µl of reaction buffer (10X)
2. 25 ng of ds DNA
3. 125 ng of oligonucleotide primer #1
4. 125 ng of oligonucleotide primer #2
5. 1 µl of dNTP mix (10 mM)
6. Sterile water added to a final volume of 50 µl
7. 1 µl of Pfu turbo DNA polymerase (2.5 U/µl) is added in the end and vortexed.

PCR program:

1. 95° C 30 sec
2. 95° C 30 sec (denaturation)
3. 55° C 1 minute (annealing)
4. 68° C 10 minutes (elongation) (repeat from step 2 for 16 cycles)
5. 4°C stop

1 µl (10U/µl) of the DpnI endonuclease was added for the digestion of the parental DNA template and incubated for 37° C for 1 h. 1 µl of the PCR mixture was used to transform the XL-1 Blue supercompetent cells and plated on LB-Ampicillin agar plates.

Two stage PCR protocol for deletion mutations:

Stage 1:

Reaction mixture 1:

1. 5 µl of reaction puffer (10X)
2. 100 ng of ds template DNA
3. 125 ng of deletion mutation oligonucleotide primer #1
4. 1 µl of dNTP (10 mM)
5. Sterile water added to a final volume of 50 µl

1 µl of pfu turbo DNA polymerase (2.5 U/µl) is added in the end and vortexed.

Reaction mixture 2:

1. 5 µl of reaction puffer (10X)
2. 100 ng of ds template DNA
3. 125 ng of deletion mutation oligonucleotide primer #2
4. 1 µl of dNTP (10 mM)
5. Sterile water added to a final volume of 50 µl

1 µl of pfu turbo DNA polymerase (2.5 U/µl) is added in the end and

vortexed.

PCR program:

1. 95° C 30 sec
2. 95° C 30 sec (denaturation)
3. 55° C 1 minute (annealing)
4. 68° C 14 minutes (elongation) (repeat steps 2-4 for 5 cycles)
5. 4° C stop.

Stage 2:

1. 50 µl of Reaction mixture 1
2. 50 µl of Reaction mixture 2

was combined and subjected to PCR reaction.

PCR program:

1. 95° C 30 sec
2. 95° C 30 sec (denaturation)
3. 55° C 1 minute (annealing)
4. 68° C 14 minutes (elongation) (repeat steps 2-4 for 16 cycles)
5. 4° C stop.

1 µl (10U/µl) of the DpnI endonuclease was added for the digestion of the parental DNA template and incubated for 37° C for 1 h. 1 µl of the PCR mixture was used to transform the XL-1 Blue supercompetent cells and plated on LB-Ampicillin agar plates. The plasmids with desired mutations were transformed into bacterial cells/ transfected into insect cells, proteins expressed (2.2.1 and 2.2.2 section) and used for further analytical experiments.

2.2.4. Construction of chimeric human and murine 12(R)-LOX:

A region spanning about 500 bp was identified between human and murine 12R-lipoxygenase, which contains 3 crucial amino acid differences that may affect the substrate specificities between the two enzymes towards arachidonic acid and its methyl ester. This region of human 12R-LOX was reverse transcribed from the HaCaT cells, a human keratinocyte cell line, and the cDNA was inserted into the same region in the murine 12R-LOX to form a chimera. Two restriction sites, Bsu36I and SphI flank the 500-bp region in the mouse 12R-LOX while in the human 12R-LOX the SphI restriction is absent. So primers were designed so as to insert a SphI restriction site in human 12R-LOX during the PCR reaction. It facilitates easy restriction site cleavage and insertion of the 500 bp region into mouse 12R-LOX.

2.2.4.1.RNA extraction and Reverse transcription of the total RNA from HaCaT cells:

RNA extraction:

Total RNA from HaCaT cells was extracted according to instructions of the Rneasy Minkit. The RNA was then reverse transcribed to get the first strand cDNA.

Reverse transcription-PCR (RT-PCR):

Total RNA (3µg) - 3.3 µl
Oligo dT18 (0.5 µg/µl) – 3.0 µl
Sterile water is added to make up to 21 µl and was heated for 72 min for 4 min.

Mix I: dNTPs (10 mM) – 3.0 µl
5 X AMV-buffer – 9.0 µl

BSA (10 µg/µl) – 0.45 µl

RNasin (RNAse inhibitor) (4 U/µl) - 0.75 µl

Water- 10.2 µl

Total volume 23.4 µl

RNA mixture-21 µl + Mix I + 0.5 µl M-MLV reverse transcriptase (Promega).

PCR program:

1. 37° C	2 h
2. 94° C	10 min
3. 4° C	stop

Polymerase Chain Reaction:

25µl BD Advantage 2 PCR buffer (10X)

5 µl dNTP Mix (50X)

5 µl BD Advantage 2 polymerase mix (50X)

2 µl H12RT up primer (5µM)

2 µl H12RT down primer (5µM)

3 µl cDNA

36 µl water to make up the volume to 50 µl

PCR program:

1. 95° C	1 min.
2. 95° C	30 sec
3. 68° C	3 min (repeat step 2 for 35 times)
4. 68° C	3 min
5. 4° C	stop

GAPDH-PCR:

35.75 µl water
5 µl PCR-puffer (10X)
2.5 µl MgSO4
2 µl F-primer
2 µl R-primer
1 µl dNTP
0.75 µl pyra taq.
49 µl + 1 µl cDNA.

PCR program:

1. 94° C	2 min	
2. 94° C	40 sec	
3. 65° C	1 min	
4. 72° C	2 min	(repeat from step 2 for 30X)
5. 72° C	8 min	
6. 4° C	stop	

The PCR products were cloned into PCR II-TOPO cloning vectors. The clones containing the segment of human 12R-LOX gene were selected and the plasmid DNA was purified. The purified DNA and the murine 12R-LOX DNA was restriction digested between Bsu36I and SphI and the fragments were ligated. The mixture was transformed into TOP 10 competent cells, plated on LB-agar and the clones formed were selected for the chimeras.

2.3. ANALYTICAL METHODS:

2.3.1. Estimation of murine 12(R)-LOX activity by HPLC:

Bacterial cell lysate or insect cell lysate which expressed 12R-LOX protein (wild type and mutants) was added to a total volume of 0.5 ml of phosphate buffered saline. The substrate concentration was 0.1 mM (final concentration) for all the fatty acid substrates tested. The mixture was vortexed and incubated for 15 min at 37° C. After the incubation time, hydroperoxy fatty acids formed in the reaction were reduced to their more stable hydroxyl derivatives by addition of sodium borohydride. After 5 min the reaction was acidified to pH 3 with 50 µl of acetic acid and then the proteins were precipitated with the addition of 0.5 ml of methanol. The total mixture was centrifuged (14000 rpm for 15 min) to remove the proteins and the clear supernatant was analysed on Reverse phase HPLC (RP-HPLC).

High-pressure liquid chromatography was performed on a Shimadzu system equipped with Hewlett-Packard diode array detector 1040A and HP-Chemstation program. Reverse phase HPLC was carried out on a Nucleosil C-18 column (250 x 4 mm, 5-µm particle size, Macherey-Nagel, Düren, Germany) and with a pre-column (30 x 4 mm). The solvent system used was methanol: water: acetic acid in the ratio of 80: 20: 0.1 with a flow rate of 1 ml/min. The chromatograms were followed by monitoring the absorbance at different wavelength:

- 235 nm for conjugated diene [hydro (pero) xy fatty acids]
- 242 nm for double conjugated dienes [DiH (p) ETEs]
- 210 nm for polyenoic fatty acids
- 270 nm for conjugated trienes

The product peaks from RP-HPLC were identified by co-injections with authentic standards under identical experimental conditions. The amounts of products (HETEs and HETE methyl esters) formed were quantified by establishing an calibration curve of conjugated dienes, conjugated trienes and linoleic acid.

2.3.2. Product Analysis:

The products prepared by RP-HPLC were further analysed by straight phase-HPLC (SP-HPLC), chiral phase-HPLC (CP-HPLC) and gas chromatography/mass spectrometry (GC/MS).

2.3.2.1. Straight phase HPLC (SP-HPLC):

SP-HPLC was performed on a Nucleosil 100-7 column (250 x 4 mm, Machery-Nagel, Düren, Germany) using a diode array SPD-M6A-detector (Shimadzu). The solvent system used was n-hexane: isopropanol (1% or 2% isopropanol) with 0.1%: acetic acid and a flow rate of 1 ml/min was adjusted.

2.3.2.2. Chiral phase HPLC (CP-HPLC):

Enantiomer composition of the LOX products can be quantified by CP-HPLC. The Chiral columns used were Chiralcel OD or OB, 250 x 4.6 mm (Diacel Chem. Industries, USA) with solvent system n-hexane/2-propanol (with different concentrations depending upon the products) and 0.1% acetic acid. The flow rate was 1 ml/min.

2.3.2.3. ***Gas Chromatography/Mass Spectrometry:***

The structure of the LOX products, for which we did not have authentic standards, was analysed with GC/MS. For this purpose the products were first derivatized to enable gas chromatographic separation. Free

carboxylates were methylated with diazomethane and hydroxyl groups were silylated with bis- (trimethylsilyl)-trifluroacetamid in dry pyridine. The measurements were carried out on a Shimadzu GC/MS QP-2000 system equipped with a fused silica Capillary SPB 1 (12m x 0.3 mm; coating thickness 0.33 µm). The injector temperature was at 270°C, the ion source temperature at 180°C and electron energy of 70 eV was used. The derivatized compounds were eluted isothermically at 180 °C.

2.3.3. Protein Quantification:

For estimating the protein concentrations, Roti-Quant System (Roth, Karlsruhe, Germany), based on Bradford method was used. For standard graph, BSA protein was used.

2.3.3.1. Polyacrylamid-Gel Electrophoresis:

For SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) the Laemmli method (Laemmli, 1970) was followed. A 12% polyacrylamide Mini-Gel was used and electrophoresis was carried out at 27-30 mA. After electrophoresis the gel was used for Western blot.

2.3.3.2. Western Blot :

Proteins separated on SDS-PAGE were blotted on to a nitrocellulose membrane by semi-dry blotting method. The membrane was blocked with 5% milk powder in PBS / 0.1% TWEEN-20 and then incubated with the primary antibody for 1 hr. After washing extensively with PBS/TWEEN it was incubated with the secondary antibody, which has peroxidase conjugate. After washing, the blot was kept in Western-Lightning Chemiluminescence Plus Reagent and exposed to X-ray film for 1s to 20 s and developed. For

quantitative analysis the intensity of the bands were estimated with the help of a program Phoretix 1D (Phoretix International, Newcastle, UK).

RESULTS

3.1. RECOMBINANT EXPRESSION OF THE MURINE 12(R)-LOX AND ENZYME CHARACTERIZATION

3.1.1. Functional expression of murine 12(R)-lipoxygenase in *E.Coli*:

The murine 12R-lipoxygenase cDNA was sub-cloned into pQE-80L vector and M15 bacterial cells were transformed. Expression of the recombinant enzyme (his-tag fusion protein) was induced with 1mM IPTG, the bacterial cells were lysed and the crude lysate supernatants were checked for the activity. Compared with rabbit 15-LOX (0.15 mg HETE/15 min/ml culture lysis supernatant) expression of the murine 12R-LOX was low (0.01 mg HETE/15 min/ml lysis supernatant). This difference was confirmed on the protein level as indicated by immunoblotting using a his-tag specific antibody. Low-level expression of the recombinant enzyme was also indicated by the fact that there were no major alterations in the pattern of the expressed proteins when the LOX-transformed bacteria were compared with *E. coli* cells transformed with the wild-type plasmid as detected by Coomassie staining following SDS-PAGE. However, in immunoblotting only LOX expression was observed when the cells were transformed with the recombinant plasmid (Fig. 10).

Next, attempt was made to purify the recombinant his-tagged protein on the Ni-NTA column in parallel with rabbit 15S-LOX. Unfortunately, the his-tagged murine 12R-LOX did not bind efficiently to the Ni-agarose column. Only less than 50 % of the activity applied was retained on the column. The bound share of the enzyme was then eluted using an increasing gradient of imidazole and two major LOX containing fractions (fractions 2 and 3) were obtained (Fig. 10). In contrast, with the rabbit 15-LOX 100 % of the activity was bound on the column. Moreover, it is found that the rabbit enzyme was bound more strongly to the matrix. The majority of the activity

was eluted later (fractions 4 and 5) than the 12R-LOX. Several attempts were made to optimise the purification procedure of 12R-LOX:

i) variations of the gel/supernatant ratio to avoid overload of the column

ii) alterations in binding and elution pH

iii) purification in the presence of 6 M urea (denaturing conditions)

Unfortunately, none of these attempts were really successful. Hence it was decided to characterise the recombinant enzyme in the crude lysis supernatant.

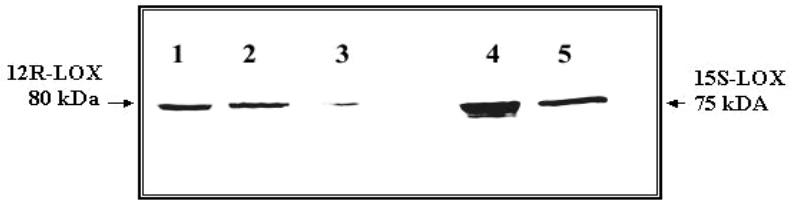


Fig. 10. Expression of recombinant murine 12R-lipoxygenase and rabbit reticulocyte 15S-lipoxygenase in bacterial cells. Bacterial cells were transformed with pQE-80L vector carrying murine 12R-lipoxygenase and pQE-30 carrying 15S-lipoxygenase cDNAs. The respective enzymes were purified on Ni-Agarose column and the eluted fractions (50 µg protein) were electrophorized on 12% SDS-PAGE. The proteins were electroblotted onto a nitrocellulose membrane and incubated with anti-his-tag antibody. Lane 1: purified fraction #2, Lane 2: fraction #3, Lane 3: fraction #4 of murine 12R-lipoxygenase, Lane 4 and Lane 5: purified fraction #4 and purified fraction #5 of rabbit reticulocyte 15S-lipoxygenase.

As expected from previous studies, incubation of crude lysis supernatant with 100 µM of free arachidonic acid did not reveal significant amounts of LOX products as indicated by HPLC analysis. However, when the corresponding methyl ester was used the formation of 12-HETE methyl ester and its hydrolysis product, free 12-HETE were observed. In addition, minor amounts of 8-HETE methyl ester and free 8-HETE as well as 11-HETE methyl ester and free 11-HETE were also detected (Fig. 11, left panel). When the partially purified enzyme obtained after Ni-agarose purification was used in the reaction mixture the free HETE isomers were not detected. Thus, formation of free HETEs was most likely due to a lipase/esterase activity

present in the bacterial lysates. The structures of the oxygenated products were identified by co-migration with authentic standards on RP- and SP-HPLC. When linoleic acid methyl ester was used as the substrate, 9-HODE methyl ester was the major product with a 9-HODE /13-HODE ratio of about 9:1.

3.1.2. Chirality of the reaction products:

Chiral phase HPLC of the purified reaction products indicated a high degree enantioselectivity of the 12R-LOX reaction with both, methyl arachidonate (Fig. 11, right panel) and methyl linoleate (not shown). In both cases we observe exclusive formation of the R-isomers.

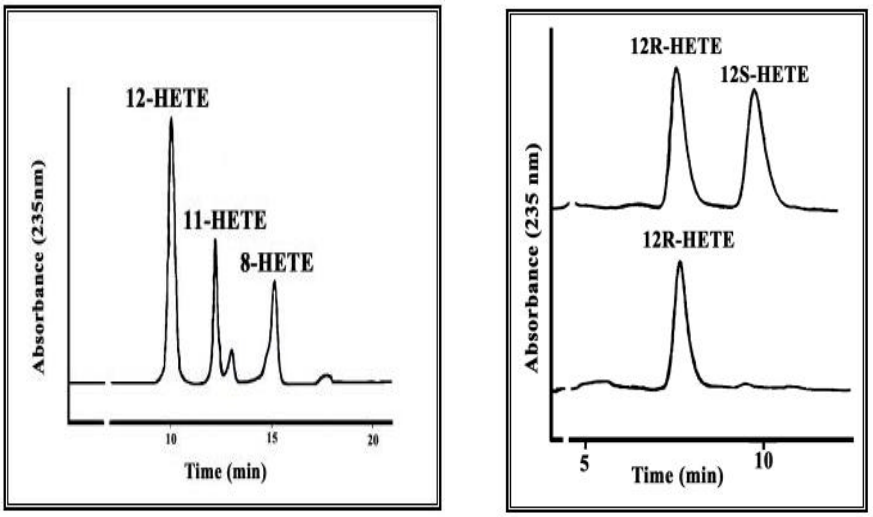


Fig.11. Straight-phase and chiral-phase HPLC chromatograms of 12R-lipoxygenase with arachidonic acid methyl ester as substrate: Left panel: Straight- phase analysis was done with HETE fractions (HETE methyl ester fraction was hydrolysed) obtained from RP-HPLC column. The column used was Nucleosil 100-7 column (250x4 mm, Machery-Nagel, Düren, Germany) with SPD-M6A-Detector (Shimadzu). The detection of the products was at 235 nm. The solvent system used was *n*-Hexane: Isopropanol (2% isopropanol) with 0.1%: acetic acid. The flow rate was 1 ml/min. **Right panel**: Chiral-phase analysis of regioisomeric HETEs separated on SP-HPLC. The 12- HETE eluate was subjected to chiral phase chromatography on a Chiralcel column, 250x4.6 mm, (Diacel Chem. Industries, USA) with solvent system *n*-Hexane/2-Propanol (2%) and 0.1% acetic acid. The flow rate was 1 ml/min and the detection was with SPD-M6A Detector (Shimadzu) with absorbance at 235 nm.

3.1.3. Purification of murine 12(R)-lipoxygenase from insect cells:

To improve the level of expression and to optimise the purification procedure, the murine 12R-LOX was next expressed in insect cells. The 12R-LOX cDNA with his-tag was subcloned into the pVL-1392 transfer vector and Sf9 cells were co-transfected with the recombinant transfer vector and BaculoGold DNA. Homologous recombination between the recombinant 12R-LOX-pVL1392 and the Baculogold DNA yielded recombinant virus, which then infected the insect cells. The kinetics of 12R-LOX expression revealed maximal activities at 72 h after infection. At this time point the cells were lysed, the lysis supernatant was applied to Ni-agarose column and the LOX protein was eluted with an increasing gradient of imidazole. The different fractions were checked for the lipoxygenase activity using our standard assay system with arachidonic acid methyl ester as substrate. Although the specific activity of the 12R-LOX expressed in the insect cell system was about 3-fold higher than that obtained in bacteria (Table 2) we did not obtain a pure LOX preparation.

Sample	LOX-activity (µg HETE/ml)	LOX protein (mg/ml)	Specific Activity (µg HETE/mg protein)
Bacterial lysate	0.93	0.093	10
Insect cell lysate	1.8	0.047	38

Table 2. Comparison of the specific activities of the 12R-LOX expressed in the bacterial cells and in the insect cells. The recombinant enzyme was expressed in 1 litre culture of bacteria and in $15x10^6$ Sf9 cells. The supernatants from the lysed cells were incubated in PBS buffer with 100 µM arachidonic acid methyl ester for 15 min at 37^0 C. The hydroperoxy derivatives of the fatty acids were reduced and then analysed on RP-HPLC. Activities were measured by comparison of the areas of the HETE and HETE methyl ester peaks with standards. The total protein used for bacterial lysates is 1.5 mg/ml and for insect lysates 0.15 mg/ml and the amount of 12R-LOX protein was calculated from the Western blot analysis. The specific activity is given in µg HETE formation/mg LOX protein during a 15 min incubation period.

With respect to purification of the recombinant protein, the same problem was

encountered in the insect cells as in bacterial lysates. Binding of the recombinant his-tag protein to the Ni - agarose was insufficient and the recombinant protein was early eluted at low imidazole concentrations when compared with the rabbit enzyme (Fig. 12).

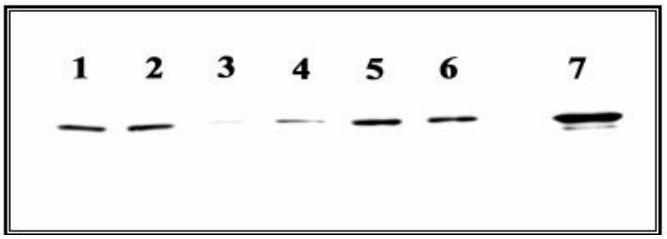


Fig.12. Western blot analysis of the Ni-agarose purification of the murine 12R-lipoxygenase expressed in insect cells. Insect cells were co-transfected with pVL 1392-12(R)-LOX and BaculoGold DNA. After infection, the insect cells were lysed and the clear lysate was passed through Ni-Agarose column and the eluted fractions (10 µg protein) were electrophorized on 12% SDS-PAGE. The proteins were electroblotted onto a nitrocellulose membrane and incubated with an anti-his-tag antibody. Lane 1: crude lysate, Lane 2: supernatant after passing through Ni-Agarose column, Lane 3: wash through, Lane 4: purified fraction #2, Lane 5: purified fraction #3, Lane 6: fraction # 4 and Lane 7: known concentration (1 µg) of rabbit reticulocyte 15(S)-lipoxygenase used as standard.

When the total insect lysates were incubated with the arachidonic acid methyl ester and then analysed by RP-HPLC two peaks were observed, which co-chromatographed with authentic standards of free and esterified 12-HETE. Thus, in the insect cell lysates too unspecific esterases hydrolyse the LOX products formed from methyl arachidonate. SP-HPLC indicated major formation of 12-HETE (69%) and minor peaks of 8-HETE (15%) and 11-HETE (11%) and the predominant formation of the R-isomer (data not shown). In summary one can conclude that the reaction specificity of the 12R-LOX was comparable regardless whether the recombinant enzyme was expressed in *E. coli* or in the baculovirus insect cell system.

Since the murine 12R-LOX does not oxygenate free polyenoic fatty acids the natural substrates for this isoform remains unknown. To shed some light on this topic we tested the substrate specificity of the enzyme expressed in the insect cell system using the following fatty acid derivatives: free

arachidonic acid and its methyl ester, free linoleic acid and its methyl ester, various hydroxylated fatty acids (16-HETE, 19-HETE, 20-HETE) and their methyl esters, dicarboxy arachidonic acid, its monomethyl and dimethyl esters. The enzyme was found to react only with the methyl esters of the substrates and there was very low activity with the free acids. From the Table 3 below, we observe that the enzyme shows more activity towards the methylated substrates than to their free acids. Among the substrates tested arachidonic acid methyl acid has the optimum activity followed by the 20-HETE methyl ester and linoleic acid methyl ester.

Substrate	activity (µg HETE/ml)	Substrate	activity (µg HETE/ml)
Arachidonic acid	0.32	**19-HETE**	0.03
Arachidonic acid methyl ester	1.80	**19-HETE methyl ester**	0.36
Linoleic acid	0.20	**20-HETE**	0.05
Linoleic acid methyl ester	1.03	**20-HETE methyl ester**	1.27
16-HETE	0.100	**Mono-methyl dicarboxy arachidonic acid**	0.074
16-HETE methyl ester	0.585	**Di-methyl dicarboxy arachidonic acid**	0.12

Table 3. Comparison of activities of 12R-LOX enzyme with different substrates. The insect cell lysates were used as enzyme source. The supernatants from the lysed cells were incubated in PBS buffer with 100 µM of substrate concentration for 15 min at 37 ^{o} C. The hydroperoxy derivatives of the fatty acids were reduced and then analysed on RP-HPLC.

SUMMARY OF THE RESULTS OF SECTION 3.1:

The 12R-LOX cDNA subcloned in pQE-80L and pVL 1392 plasmids were transformed and transfected into bacterial and insect cells respectively. We could express the 12R-LOX successfully in both the expression systems.

- The specific activity in insect cells was found to be higher than that in bacterial cells (about 4X higher).
- The major product formed from the enzyme expressed in both the systems with arachidonic acid methyl ester is 12-HETE methyl ester (70%) which when analysed on CP-HPLC was found to be exclusively R-isomer (98%).
- The minor products formed were 11-HETE methyl ester (11%) and 8-HETE methyl ester (15%).
- The major products formed with linoleic acid methyl ester as substrate are 9R-HODE (90%) and 13S-HODE (10%).

SITE-DIRECTED MUTAGENESIS STUDIES

3.2. DETERMINANTS FOR POSITIONAL SPECIFICITY:

Previous mutagenesis studies on various S-lipoxygenases have identified four major sequence determinants for the positional specificity of lipoxygenases and they are named according to their discoverer (Sloane determinants, Borngräber I and Borngräber II determinants). In order to establish a comprehensive theory on the structural basis of the positional specificity of LOXs we intended to test whether these amino acids may also be of importance for the murine 12R-LOX. For this purpose these sequence determinants were targeted by site directed mutagenesis and the positional specificity of the mutant enzyme species was analysed. To initiate these studies we first identified the target amino acids by multiple sequence alignments. These alignments indicated the following amino acids to be mutated: A455 (*Sloane determinant*), F390 (*Borngräber I determinant*) and V631 (*Borngräber II determinant*). According to the "space-related" model of the positional specificity, a 12-LOX can be converted to a 15-LOX when the volume of the active site is reduced. This concept has been tested for human platelet-type 12-LOX, the porcine leukocyte-type 12-LOX, the rabbit

reticulocyte 15LOX and the human reticulocyte-type 15-LOX (15-LOX1). To test this concept for the murine 12R-LOX more space-filling amino acids were introduced at these positions.

3.2.1. Phenylalanine390 (*Borngräber I Determinant*):

When Phe-353 was mutated in rabbit 15S-LOX to less space-filling amino acid the mutant enzyme exhibited arachidonate 12-lipoxygenase activity (Borngräber et al. 1996). Sequence alignments of the known mammalian 12/15-LOXs indicated that enzyme species carrying a small residue at position 353 are 12-LOXs regardless of the side chain geometry of other sequence determinants for the positional specificity (Borngräber et al. 1996). If there is a space filling amino acid at position 353, the bulkiness of amino acids 417 and 418 determine whether the enzyme catalyses 12- or 15-lipoxygenation (Borngräber et al. 1996). Thus, 15-lipoxygenating enzyme species contain a bulky residue at 353 and space-filling side chains at 417/418. In contrast, 12-LOXs contain either a small residue at position 353 or, if they have a bulky side-chain at 353, small residues are located at positions 417/418. In the murine 12R-lipoxygenase there is the same bulky Phe at the position that aligns with Phe353 of the rabbit enzyme and a small Ala (A455) at the position, which aligns with Ile418 (see 3.2.2.). This constellation predicts a 12-lipoxygenation and according to the rules explained above there should be a still smaller space for converting the murine 12R-LOX to a 15-lipoxygenating enzyme as the residues aligning with Ile417/Met418 appear to be more important (see 3.2.2.).

Mouse 8(S)-LOX								
R N	S	E	**F**	Y	I	H	E	370
Mouse 12(R)-LOX								
R Y	A	E	**F**	Y	S	H	E	394
Mouse 5(S)-LOX								
R S	S	D	**F**	H	V	H	Q	364
Rabbit 15(S)-LOX								
R S	S	D	**F**	Q	V	H	E	357

Fig.13. Sequence alignments of various LOX-species surrounding the Borngräber I determinant for the positional specificity.

From Fig. 13 it can be seen that Phe 390 of the murine 12R-LOX aligns with Phe353 of the rabbit enzyme. The only possibility to decrease the volume of the substrate-binding pocket and thus, to force conversion of 12R-LOX to a 15-lipoxygenating mutant was a Phe-Trp exchange since Trp is the only proteinogenic amino acid carrying a more bulky side chain than Phe. Unfortunately, this mutant was catalytically inactive. Next, we applied an inverse strategy and introduced smaller amino acids at position 390. Although this strategy did not follow the space hypothesis of positional specificity it was interesting to see whether or not such mutations may impact the positional specificity of the enzyme.

mutant	activity (µg HETE/ml)	12R-LOX protein (mg/ml)	Specific Activity (µg HETE/mg protein)
Wild Type	1.34	0.098	14
F390L	1.3	0.160	8
F390A	0.65	0.122	5

Table 4. Quantification of the specific activities of *Borngräber I determinant* mutants of the murine 12R-LOX using arachidonic acid methyl ester as substrate. The mutants were created as described in the Methods section and the bacterial cell lysates were used as enzyme source. LOX activity was determined by HPLC quantification of the reaction products. The amount of LOX protein in the lysates was quantified by Western blotting using the pure his-tag rabbit 15-LOX as mass standard. The specific activity is given in µg HETE formation/mg LOX protein during a 15 min incubation period.

When expressed in *E. coli* (Table 4) F390L and F390A were enzymatically active with arachidonic acid methyl ester as substrate. However, the specific activities were somewhat lower than that of the wild-type enzyme. Comparison of the product patterns suggests a tendency for more unspecific product formation. With the wild-type enzyme 12(R)-H(p)ETE was the main product (63%). This share was decreased to 44% for the F390L mutant and to 29 % for the F390A exchange. For the latter mutant we even observed a significant share (20%) of 5-H(p)ETE formation. This product was stereochemically pure (R:S-ratio of 99:1) indicating that its formation was completely controlled by the enzyme (Table 5).

Mutant	**Share (%)**					
	12-HETE	**15-HETE**	**11-HETE**	**9-HETE**	**8-HETE**	**5-HETE**
Wild Type	63 (92R:8S)	0	18	7	12 (82R:18S)	0
F390L	44 (98R:2S)	0	11	16	29	0
F390A	29 (86R:14S)	8	14	12	17	20 (99R:1S)

Table 5. Straight-phase and Chiral-phase analysis of the reaction products of *Borngräber I* mutants of 12R-lipoxygenase with arachidonic acid methyl ester as substrate. Straight phase analysis was done with HETE fractions (HETE methyl ester fraction was hydrolysed) of RP-HPLC. The different products separated on SP-HPLC were further analysed on CP-HPLC. Authentic standards were used as external standards. The concentrations of different products and S and R enantiomers were determined from the peak areas and are used to determine the ratios, the sum of which was set to 100%.

3.2.2. Alanine455 (*Sloane Determinant*):

Comparing conserved sequences of 12- and 15-lipoxygenases, a small region responsible for functional differences between 12- and 15-lipoxygenases has been identified. Replacement of two amino acids in the human 15-LOX (M417 and I418) by those residues found in 12(S)-LOXs resulted in an enzyme with major 12-LOX activity (Sloane at al., 1991). For

the rabbit 15-LOX1, Ile418Ala exchange was sufficient to completely convert this enzyme to a 12-lipoxygenating species. For this LOX-isoform Met419 was not of major importance (Borngräber et al., 1999). When in reverse, Val418 and Val419 of porcine leukocyte 12-lipoxygenase were replaced by Ile or Met respectively, a predominant 15-lipoxygenase activity was observed (Suzuki et al., 1994).

Mouse 8(S)-LOX									
L	I	D	K	**S**	**T**	G	L	G	T
	436								
Mouse 12(R)-LOX									
L	S	A	R	**A**	**M**	S	L	G	L
	460								
Mouse 5(S)-LOX									
L	F	D	K	**A**	**N**	A	T	G	G
	430								
Rabbit 15(S)-LOX									
I	F	D	Q	**I**	**M**	S	T	G	G
	423								

Fig.14. Sequence alignments of various LOX-species surrounding the Sloane determinant for the positional specificity.

Based on the sequence alignments with 15-lipoxygenase we have identified Ala455 and Met456 as corresponding amino acids in the murine 12R-LOX. Ala455 was mutated to more space-filling residues, such as Ile, Phe and Trp and the specific activity and product profiles of the mutants were analysed.

When expressed in *E. coli* comparison of the specific catalytic activities of the mutants indicated that the amino acid exchanges introduced resulted in reduction of the enzymatic activity. This reduction was particularly evident for the A455I exchange. This result is quite surprising since among the mutations carried out the Ala-Ile exchange was the most conservative

alteration. In contrast, introduction of more bulky residues (Ph, Trp) did not lead to such a dramatic drop in the catalytic activity.

Mutant	LOX activity (µg HETE/ml)	LOX protein (mg/ml)	Specific Activity (µg HETE/mg protein)
Wild type	0.959	0.095	10
A455I	0.100	0.109	1
A455F	0.845	0.156	5.6
A455W	0.52	0.100	4.5

Table 6. Quantification of the specific activities of *Sloane determinant* mutants of the murine 12R-LOX using arachidonic acid methyl ester as substrate. The mutants were created as described in the Methods section and the bacterial cell lysates were used as enzyme source. LOX activity was determined by HPLC quantification of the reaction products. The amount of LOX protein in the lysates was quantified by Western blotting using the pure his-tag rabbit 15-LOX as mass standard. The specific activity is given in µg HETE formation/mg LOX protein during a 15 min incubation period.

Comparison of the product patterns of the different enzyme species indicated that 12R-H(p)ETE was the major product for the wild-type enzyme and its A455F mutant. A455I and A455W exchange led to a loss in product specificity. Although 11(R)- and 12(R)-H(p)ETE, respectively, were the major products for these mutants their relative shares were rather small (33% in both cases) and other HETE isomers contributed similar amounts to the sum of the oxygenation products. In summary one may conclude that A455F exchange did not impact the product pattern of the murine 12R-LOX but A455I and A455W mutation rendered the LOX to a more unspecific enzyme. Thus, mutation of the amino acid that aligns with the Sloane determinant of the positional specificity did not lead to specific alterations in the positional specificity of the enzyme.

mutant	share (%)					
	12-HETE	**15-HETE**	**11-HETE**	**9-HETE**	**8-HETE**	**5-HETE**
Wild-type	63% (92R:8S)	0	18%	7%	12% (82R:12S)	0
A455I	23% (98R:2S)	10%	33% (98R:2S)	21%	13%	0
A455F	78%	0	10%	0	12%	0
A455W	33% (86R:14S)	8%	12% (60R:40S)	14%	17%	16% (49R:51S)

Table 7. Quantification of the product pattern of *Sloane determinant* mutants of the murine 12R-LOX using arachidonic acid methyl ester as substrate. The mutants were created as described in the Methods section and the bacterial cell lysates were used as enzyme source. LOX products were prepared and analysed by sequential RP- and SP-HPLC. Enantiomer composition of main products (values given in parenthesis) was quantified by CP-HPLC (see methods section).

3.2.3. Valine631 (*Borngräber II*):

The crystal structure of the rabbit reticulocyte 15-lipoxygenase predicted that Ile593, sitting near the base of the substrate binding pocket, may play a role in the alignment of arachidonic acid at the active site and thus, may influence the positional specificity of the enzyme (Gillmore et al., 1997). Thus, replacement of Ile593 with Ala was predicted to increase the volume of the substrate-binding pocket favouring arachidonic acid 12-lipoxygenation. This hypothesis was confirmed by site-directed mutagenesis since the major reaction products of arachidonic acid oxygenation by the I593A mutant of the rabbit 15-LOX were 15S-HETE and 12S-HETE in a ratio of 57/43, compared with 97/3 for the wild type enzyme (Borngräber et al. 1999). These results suggest that substitution of the branched Ile with the smaller Ala increases the volume of the substrate-binding pocket allowing the substrate to penetrate deeper into the active site.

Mouse 8(S)-LOX									
Y	H	I	I	**A**	L	W	L	L	612
Mouse 12(R)-LOX									
I	V	L	L	**V**	L	W	T	L	635
Mouse 5(S)-LOX									
W	H	L	G	**A**	V	W	A	L	608
Rabbit 15(S)-LOX									
L	Q	M	S	**I**	V	W	Q	L	593

Fig.15. Sequence alignments of various LOX-species surrounding the Borngräber II determinants for the positional specificity.

The *Borngräber II* determinant in the 12(R)-LOX was identified as Val631 by multiple sequence alignments (Fig. 15). To reduce the volume of the substrate binding pocket we first mutated Val631 to more space-filling amino acids like Ile and Phe to force a substrate alignment at the active site, which is optimal for 15-lipoxygenation (C-13 is localized in close proximity to the non-heme iron so that hydrogen can be removed from this bisallylic methylene). Inversely, we increased the volume of the substrate-binding pocket by introducing less space filling amino acids (Ala, Gly) at this position.

Mutant	**LOX activity (µg HETE/ml)**	**12R-LOX protein (mg/ml)**	**Specific Activity (µg HETE/mg protein)**
Wild-type	0.93	0.093	10
V631F	0.04	0.100	0.4
V631I	0.35	0.143	2.4
V631A	2	0.081	25
V631G	2.58	0.100	26

Table 8. Quantification of the specific activities of *Borngräber II determinant* mutants of the murine 12R-LOX using arachidonic acid methyl ester as substrate. The mutants were created as described in the Methods section and bacterial lysates were used as enzyme source. LOX activity was determined by HPLC quantification of the reaction products. The amount of LOX protein in the lysates was quantified by Western blotting using the pure his-tag rabbit 15-LOX as mass standard. The specific activity is given in µg HETE formation/mg protein during a 15 min incubation period.

When expressed in *E. coli* all mutants created except for the V631F exchange were enzymatically active. Interestingly, the mutants containing less space-filling residues (V631A and V631G) exhibited an increased specific activity when compared with the wild-type enzyme.

Next, we analysed the product specificity of the different Borngräber II mutants using arachidonic acid methyl ester as substrate. V631F was not analysed since the specific activity was less than 5 % of that of the wild-type enzyme. V631I exchange, which is supposed to decrease the volume of the substrate-binding pocket led to a loss of the reaction specificity of the enzyme. In fact, this mutant produced almost similar amounts of 12-. 15-, 11-, 9-, and 8-H(p)ETE. When the V631A and V631G mutants were incubated with arachidonic acid methyl ester 11-HETE was the major reaction product (Fig. 16). Its share was more than doubled when compared with the wild-type enzyme. In contrast, the share of 12-HETE, which constituted the major reaction product for the wild-type enzyme, was strongly reduced. Analysis of the enantiomer composition of 11-HETE revealed a high degree of optical purity with strong preponderance of the R-isomer while that of 12-HETE was found to be racemic (Fig. 17).

In Table 9 the composition of the product patterns of the active Borngräber II mutants is summarized. As indicated in Fig. 16 and 17, 11(R)-HETE was the major product for the mutants containing a less space-filling residue at position 631 (V631A and V631G). In addition 9(R) - and 8(S)-HETE were found in larger quantities than the wild type. The V631I exchange led to an enzyme species, which converted arachidonic acid methyl ester to a rather unspecific product pattern. Similar amounts of 12-, 15-, 11-, 9- and 8-HETE were formed indicating that this mutant enzyme species is capable of catalysing hydrogen abstraction from all bisallylic methylenes of the substrate.

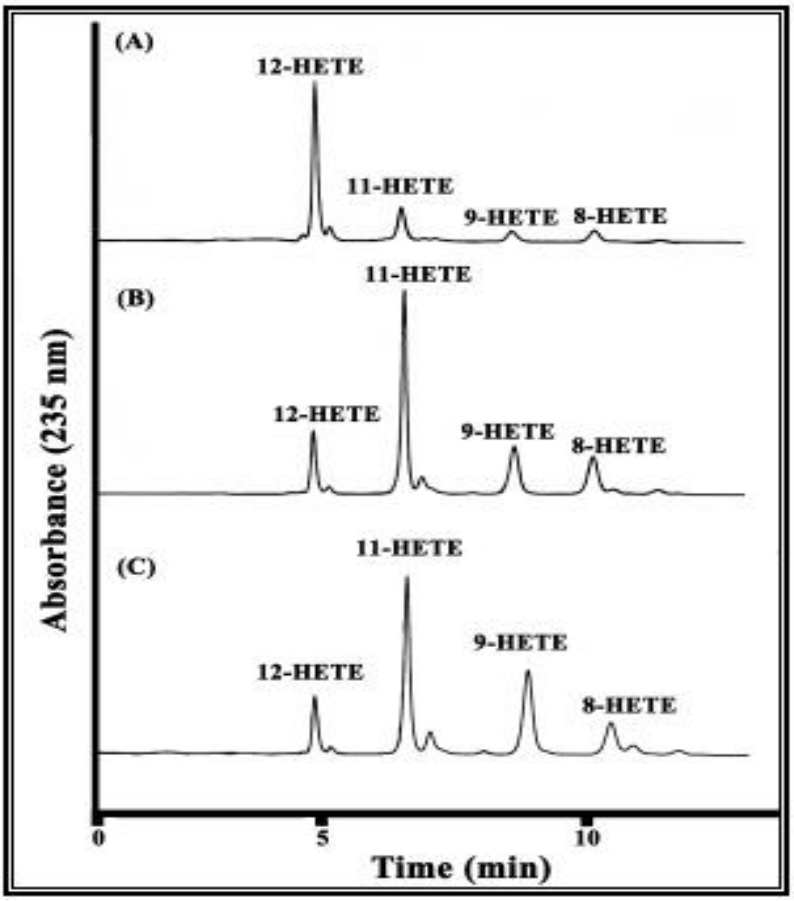


Fig.16. Straight-phase HPLC chromatograms of active *Borngräber II* mutants: Oxygenated fatty acid derivatives were purified by RP-HPLC. The methyl esters were hydrolysed, the free acids were purified by RP-HPLC and further analysed by SP-HPLC as described in the Methods section. A) Wild type, B) V631A, C) V631G.

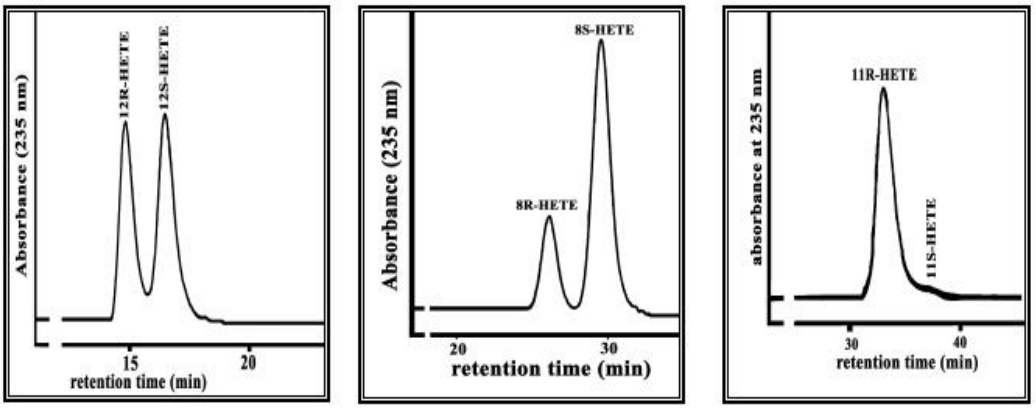


Fig.17. Chiral-phase HPLC of V631A mutant. Chiral phase HPLC of the SP-HPLC purified reaction products was carried out as described in the Methods section. Left panel: 12-HETE, middle panel: 8-HETE, right panel: 11-HETE.

Mutant	Share (%)				
	12-HETE	**15-HETE**	**11-HETE**	**9-HETE**	**8-HETE**
Wild type	63% (92R:8S)	0	18%	7%	12% (82R:12S)
V631I	20%	17%	21%	29%	12%
V631A	14% (42R:58S)	2%	49% (98R:2S)	19%	16% (21R:79S)
V631G	12% (46R:54S)	2%	42% (99R:1S)	31% (92R:8S)	13% (20R:80S)

Table 9. Quantification of the product pattern of active *Borngräber determinant* mutants of the murine 12(R)-LOX using arachidonic acid methyl ester as substrate. The mutants were created as described in the Methods section and bacterial lysate supernatants were used as enzyme source. LOX products were prepared and analysed by sequential RP- and SP-HPLC. Enantiomer composition of main products (values given in parenthesis) was quantified by CP-HPLC (see Methods section).

3.2.3.1. Expression of V631A and V631G in insect cells:

To confirm the results on the positional specificity of the Borngräber II mutants (V631A and V631G) we expressed the most interesting active mutants in the baculovirus insect cell system. For this purpose the cDNA of the two mutants were cloned into the pVL 1392 transfer vector and insect cells were co-transfected with BaculoGold DNA and the recombinant transfer vector. After 72 h, the cell supernatant, which constitutes an efficient source of the recombinant baculovirus, was collected and a fresh monolayer of insect cells were infected. 72 h post-infection the cells were prepared, lysed and the lysis supernatant was tested for LOX activity in the standard assay system using arachidonic acid methyl ester as substrate.

Mutant	LOX activity (µg HETE/ml)	12(R)-LOX protein (mg/ml)	Specific Activity (µg HETE/mg protein)
Wild Type	1.8	0.047	38
V631A	2.8	0.052	54
V631G	2.5	0.059	43

Table 10. The specific activities of the active mutants of 12(R)-LOX expressed in the Sf9 insect cells. The recombinant enzymes were expressed in $15x10^6$ cells. The supernatants from lysed insect cells were incubated in PBS buffer with 100 µM arachidonic acid methyl ester for 15 min at 37^0 C. The hydroperoxy derivatives of the fatty acids were reduced and then analysed on RP-HPLC. Activities were measured by comparison of the areas of the HETE and HETE methyl ester peaks with standards. The total protein used for the reaction is 0.15 mg/ml and the amount of 12R-LOX protein was calculated from the Western blot analysis. The specific activity is given in µg HETE formation/mg LOX protein during a 15 min incubation period.

Mutant	Share (%)			
	12-HETE	**11-HETE**	**9-HETE**	**8-HETE**
Wild type	69% (92R:8S)	11%	5%	15% (82R:12S)
V631A	13% (42R:58S)	49% (98R:2S)	27%	10% (21R:79S)
V631G	12% (46R:54S)	42% (99R:1S)	31% (92R:8S)	13% (20R:80S)

Table 11. Quantification of the product pattern of active *Borngräber determinant* mutants of the murine 12(R)-LOX expressed in Sf9 insect cells using arachidonic acid methyl ester as substrate. The mutants were created as described in the Methods section and the insect cell lysate supernatants were used as enzyme source. LOX products were prepared and analysed by sequential RP- and SP-HPLC. Enantiomer composition of main products (values given in parenthesis) was quantified by CP-HPLC (see Methods section).

As observed for the mutants expressed in bacteria, the V631A and V631G mutants in insect cells also gave 11-HETE as the major product with arachidonic acid methyl ester as the substrate (Table 11). Analysis of the enantiomer composition indicated a strong preponderance of the R-isomer. Similarly, the 8-HETE formed by the two mutants was more or less chiral with the S-isomer dominating. This is in contrast to the 8-HETE formed in the wild type enzyme where it is a R-isomer. The 12-HETE formed by the mutant enzymes as minor side product turned out to be a racemic mixture. Though the product patterns are similar between the mutants expressed in the bacterial and insect cells, there is a noticeable difference in their specific activities (Table 8 and Table 10). The specific activities of the mutants in insect cells are twice that of the enzymes expressed in bacteria. Lower activity in the bacterial cells indicates a possible involvement of post-translational modifications of the protein for the optimum enzyme activity, which can only be possible in the eukaryotic expression systems.

3.2.3.2. Reaction of the active mutants with linoleic acid methyl ester:

Except from arachidonic acid methyl ester the murine 12R-LOX does also oxygenate methyl linoleate (Siebert et al., 2001) but the reactivity with this substrate was lower than that with methyl arachidonate. We confirmed this data for the wild-type enzyme expressed in the insect cells (22 µg HODE/mg LOX protein with methyl linoleate vs. 38 µg HETE/mg LOX protein with methyl arachidonate; Table 12). However, when the most active Borngräber II mutants (V631A and V631G) were allowed to react with linoleic acid methyl ester we found a strongly augmented specific activity (Table 12). For these enzyme variants methyl linoleate appears to be the better substrate.

Mutant	LOX activity (µg HODE/ml)	protein (mg/ml)	Specific Activity (µg HODE/mg protein)
Wild Type	1.03	0.047	22
V631A	7.6	0.052	145
V631G	7.2	0.059	123

Table 12. Specific activities of the active mutants of 12R-LOX expressed in the Sf9 insect cells with linoleic acid methyl ester as substrate. The experimental conditions were the same as described in Table 8 with the exception that linoleic acid methyl ester was used as substrate. The specific activity is given in µg HODE formation/mg LOX protein during a 15 min incubation period.

Methyl arachidonate is mainly converted to 12(R)-HETE and this reaction involves hydrogen abstraction from C-10 and [+2] radical rearrangement. Linoleic acid methyl ester contains only one bisallylic methylene and thus, hydrogen can only be abstracted from C-11. Considering a [+2] radical rearrangement the formation of 13(R)-H(p)ODE was actually expected. Surprisingly, when we analysed the product pattern, 9(R)-H(p)ODE was the major product for both the wild-type enzyme and the two Borngräber II mutants (Fig. 18). In fact almost identical shares of 9- and 13-H(p)ODE were formed with these three enzyme species. Thus, with linoleic acid methyl ester as substrate no alteration in the positional specificity was observed when the Borngräber II determinants were mutated (Table 13). In contrast, with arachidonic acid methyl ester we observed prominent alterations in the positional specificity (3.2.3.1).

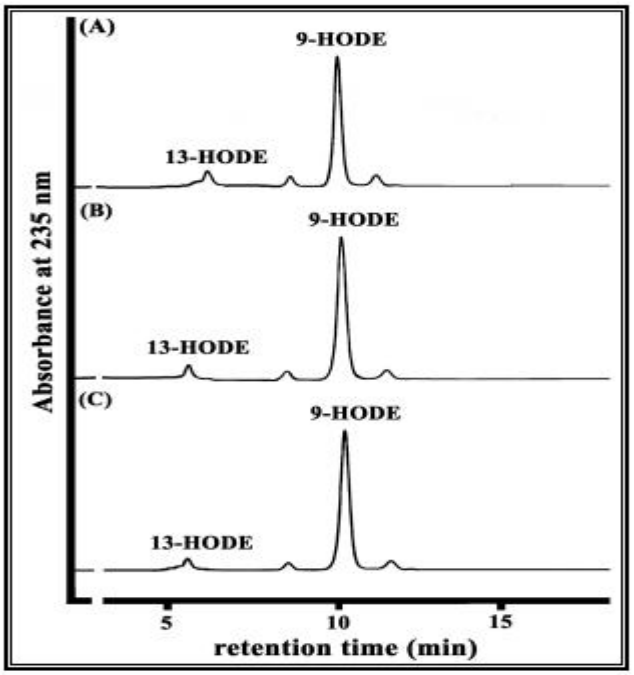


Fig.18. Straight-phase HPLC chromatograms of Borngräber II active mutants with linoleic acid methyl ester: Straight- phase analysis was performed with HODE fractions (HODE methyl ester fraction was hydrolysed) obtained from RP-HPLC column. The column used was Nucleosil 100-7 column (250x4 mm, Machery-Nagel, Düren, Germany) with SPD-M6A-Detector (Shimadzu). The detection of the products was at 235 nm. The solvent system used was *n*-hexane: isopropanol (2%) with 0.1%: acetic acid. The flow rate was 1 ml/min.

Mutant	**Share (%)**	
	9-HODE	**13-HODE**
Wild type	90% **(77R:23S)**	10% **(18R:82S)**
V631A	87% **(78R:22S)**	13% **(20R:80S)**
V631G	92% **(75R:25S)**	8% **(20R:80S)**

Table 13. Products formed from linoleic acid methyl ester by the *Borngräber II* mutants of the murine 12(R)-LOX. The different mutants were expressed in the insect cell system and aliquots of the stroma free lysates were incubated with linoleic acid methyl ester. Product preparation and analysis (SP- and CP-HPLC) were carried out as described in the Methods section R/S-ratio is given in parenthesis.

3.2.3.3. Reaction specificity with 20-HETE as substrate

It has been reported before that arachidonic acid methyl ester is the preferred substrate for the murine 12R-LOX (Krieg et al., 1999 and Siebert et al., 2001). Unfortunately, this substrate is rarely present in mammalian cells and thus, the question about the natural substrate for this LOX-isoform remained unanswered. To contribute to the search for the most suitable 12(R)-LOX substrate we tested a modified arachidonic acid derivative, 20-HETE methyl ester, as substrate for this LOX isoform. First, we determined the specific activities of the wild-type enzyme and of the Borngräber II mutant V631A with 20-HETE methyl ester. From Table 14 it can be seen that 20-HETE methyl ester was oxygenated with a similar activity as methyl arachidonate. Here again, the Borngräber II mutant V631A was more effective in oxygenating this substrate when compared with the wild-type enzyme

Mutant	**LOX activity (µgdiHETE/ml)**	**12R-LOX protein (mg/ml)**	**Specific Activity (µg diHETE/mg prot.)**
Wild-type (AA methyl ester)	2.72	0.047	58
Wild-type (20-HETE methyl ester)	2.53	0.047	54
V631A	15.93	0.052	304

Table 14. The specific activities of the active mutants of 12R-LOX expressed in the Sf9 insect cells with 20-HETE methyl ester as substrate. The recombinant enzymes were expressed in $15x10^6$ cells. The supernatants from lysed insect cells were incubated in PBS buffer with 100 µM 20-HETE methyl ester for 15 min at 37^0 C. Activities were measured by comparison of the areas of the diHETE and diHETE methyl ester peaks with standards on RP-HPLC.

The free acid of 20-HETE was not oxygenated, neither by the wild-type enzyme nor by the V631A mutant. Next, we analyzed the pattern of reaction products formed from 20-HETE methyl ester during its oxygenation by the wild-type 12R-LOX and its V631A mutant. SP-HPLC (Fig. 19) indicated three major peaks (I, II and III) and there were considerable differences in the product pattern between the two enzyme species.

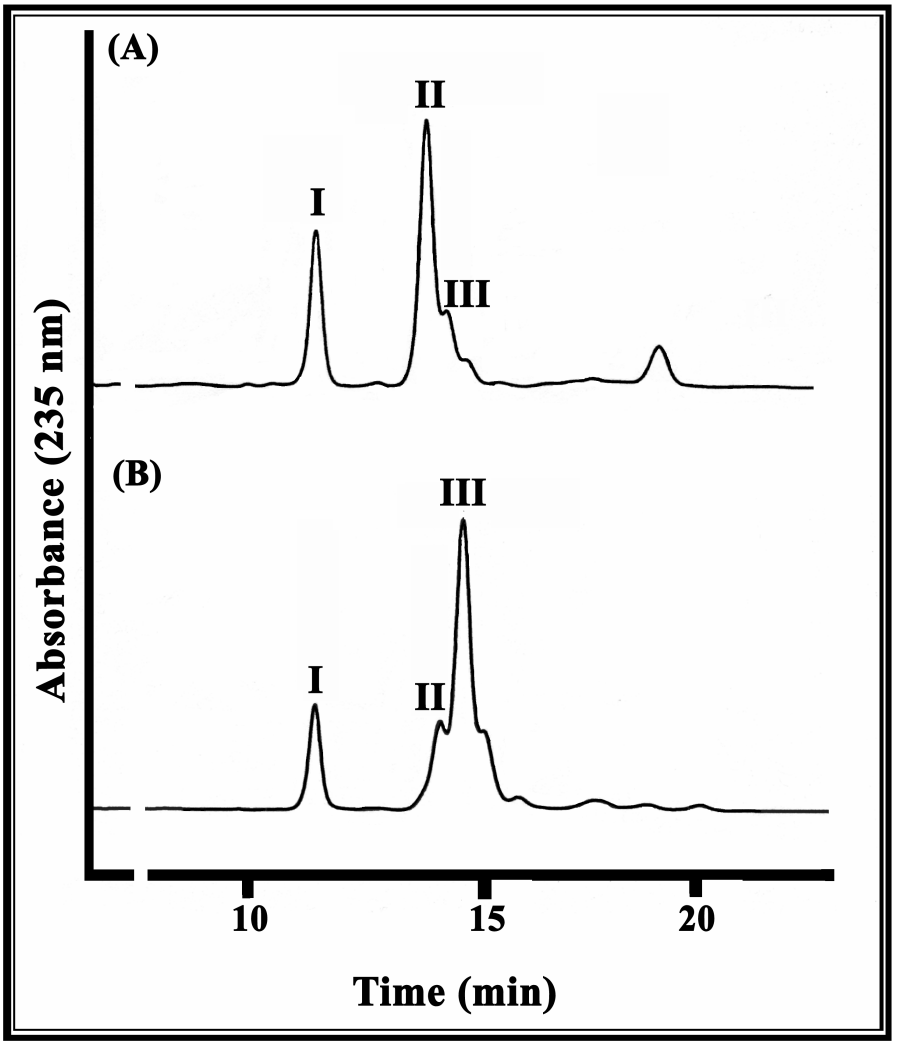
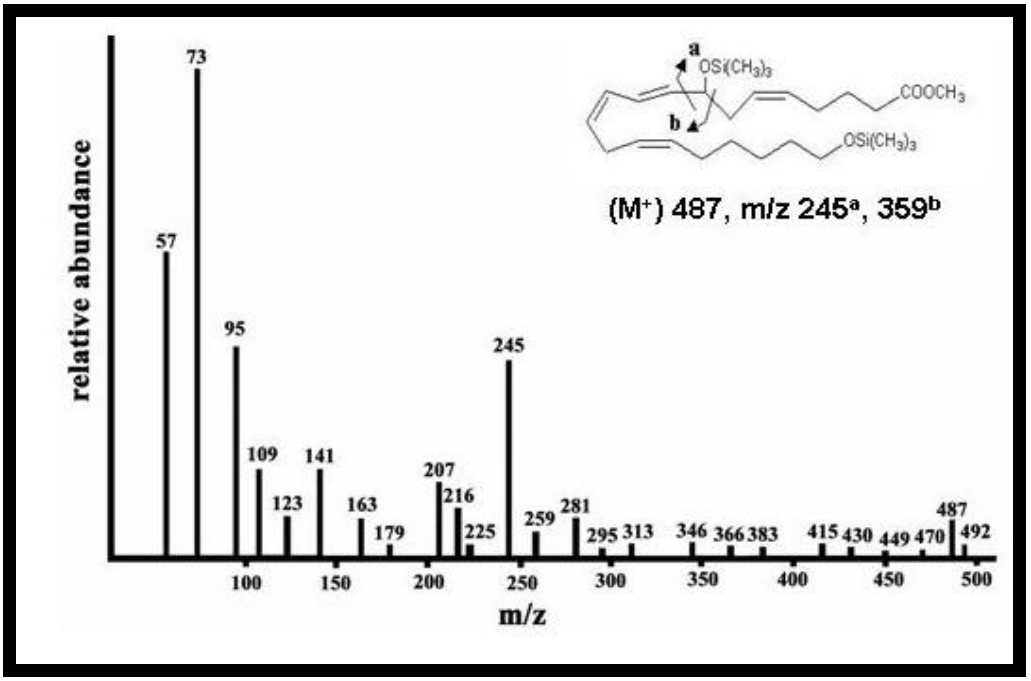


Fig.19. SP-HPLC analysis of 20-HETE-methyl ester oxygenation products. Reaction products were prepared by RP-HPLC and further analyzed by SP-HPLC on a Nucleosil 100-7 column (250x4 mm, Machery-Nagel, Düren, Germany). The solvent system used was *n*-hexane:Isopropanol (5%) with 0.1%: acetic acid; flow rate 1 ml/min. A) Wild-type, B) V631A. **Peak I** was 12,20-diHETE, **peak II** was 8,20-DiHETE, **peak III** was 9,20-DiHETE (see below).

Peak II:



Peak III:

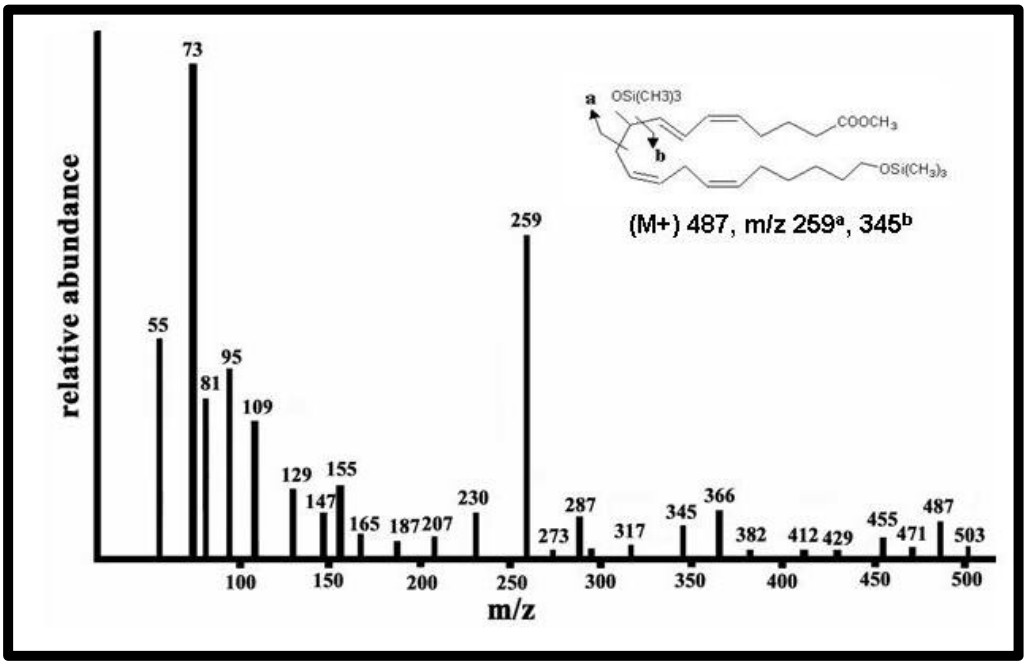


Fig.20. Mass spectral analysis of the trimethylsilyl derivatives of 8,20-DiHETE (Peak II) and 9, 20-DiHETE (Peak III). The peaks II and III collected from the SP-HPLC analysis of 20-HETE-methyl ester oxygenation products were prepared for GC/MS as described in the Methods section. The GC/MS analysis of the products was carried out on a Shimadzu GC-MS QP-2000 system equipped with a capillary column (30m x 0.32 mm, coating thickness 0.25 µm).

Peak I was identified as 12, 20-DiHETE by co-chromatography with an authentic standard. The 12, 20-DiHETE standard was prepared from 20-HETE by 12-lipoxygenation of I418L mutant of 15S-LOX. Since no authentic standards were available for the other oxygenation products of 20-HETE we collected the peaks and analysed them by gas chromatography/mass spectrometry. The corresponding mass spectra are shown in Fig. 20.

Mutant	**Share (%)**		
	12,20- di HETE	**9,20- di HETE**	**8,20- di HETE**
Wild Type	28%	14%	58%
V631A	18%	64%	18%

Table 15. Pattern of the oxygenation products formed from 20-HETE by the wild-type 12R-LOX and its V631A mutant. Reaction products were prepared by RP-HPLC and hydrolysed under alkaline conditions (see Methods section). After re-purification by RP-HPLC the free fatty acid derivatives were further analysed by SP-HPLC as described in the legend to Fig. 19.

From the above Table 15, it is clearly shown that when 20-HETE methyl ester is used as substrate for 12R-LOX there is a complete change in the product pattern. The 20-HETE methyl ester has a hydroxyl group attached to the 20 carbon and this results in the shift of product formation from the 12-HETE with the arachidonic methyl ester to major 8, 20-diHETE methyl ester with the 20-HETE methyl ester. The mutant V631A which generated majorily 11-HETE with the arachidonic acid methyl ester produced 9, 20-diHETE methyl ester with the 20-HETE methyl ester. This clearly indicates a change in the substrate orientation of the substrate in the active site. There is also minor quantity of 12, 20-diHETE formed indicating that the substrate may bind at the active site in both the directions. The presence of a hydroxyl group at the omega end of the fatty acid favours that end

entering the active site first because it is more tolerated in the hydrophobic active site than the methyl group.

SUMMARY OF THE RESULTS OF THE SECTION 3.2:

Based on the sequence alignments between murine 12R-LOX and rabbit 15S-LOX, we have identified the amino acids (F390, A455, and Ile631) which might be involved in the positional specificity of the 12R-LOX. We have mutated the residue to bigger amino acids to reduce the active site of the enzyme which would favour 15-lipoxygenation according to the space-based model hypothesis. The results which we have obtained are:

- Decreasing the space of the active site of 12R-LOX enzyme by mutating the Phe, Ala and Ile to a bigger, space occupying amino acids did not result in the shift from 12- to 15-lipoxygenation.

- When the space of the active site is increased by mutating the F390 to Leucine and Alanine there is unspecific product formation and when V631 was replaced with Alanine/Glycine there is a drastic change in the specific activities and product patterns of the mutants.

- With V631A and V631G there is an increase in the specific activity of the mutants about 2 times more than that of the wild type. There is also a change in the product pattern with 11-HETE as the major product in the mutants. The other minor products formed were 12-HETE, 9-HETE and 8-HETE.

- Chiral analysis indicated that the 11-HETE is predominantly a R-isomer while the 12-HETE was found to be racemic. With linoleic acid methyl ester as substrate, 9R-HODE was the major product both in the wild type and the mutants.

- With the modified substrate, 20-HETE methyl ester, the wild type enzyme formed a major 8, 20-diHETE while the mutant V631A formed major 9, 20-diHETE indicating a change in the substrate orientation at the active site.

3.3. DETERMINANTS FOR STEREOSPECIFICTY

3.3.1. Glycine441 (Brash Determinants):

Comparing the amino acid sequences of R- and S-LOXs it was found that the amino acid at position 355 (according to rabbit reticulocyte 15(S)-lipoxygenase) was always an alanine in S-lipoxygenases and a glycine in the R-lipoxygenases. When this Ala in the human 15-LOX2 was mutated to a Gly, arachidonic acid was oxygenated to a mixture of 15S-HETE and 11R-HETE. In contrast, with the wild-type enzyme 15S-HETE was the only oxygenation product. These data suggested that this position might be important for the positional specificity of R-LOXs. It was hypothesized that in the wild-type human 15-LOX2 the Ala side chain may sterically hinder oxygen insertion at C-11 and thus, only 15-lipoxygenation is possible. In contrast, the small Gly may also allow oxygen insertion at C-11 so that a mixture of 15S- and 11R-HETE can be formed. This hypothesis is quiet challenging since Ala and Gly do only differ by an additional CH_3-group. Hence, the spatial effect of the Gly-Ala exchange is rather subtle. To find out whether or not such amino acid exchange may impact the product pattern of the murine 12R-LOX we carried out mutagenesis studies along this line. First we identified Gly441 as target amino acid in the murine 12(R)-LOX by multiple amino acid sequence alignment and then we introduced more space filling residues (Ala, Val) at this position. For these experiments care was taken not to introduce bulky side chains into the protein in order to avoid complications with protein folding. When expressed in insect cells the G441A mutant was enzymatically active as

indicated by the formation of oxygenation products. However, its specific activity was only about 50 % of that determined under identical conditions for the wild-type enzyme (Table 16).

Mutant	LOX activity (µg HETE/ml)	12R-LOX protein (mg/ml)	Specific Activity (µg HETE/mg protein)
Wild Type	4.3	0.0335	128
G441A	2.15	0.0337	64
G441V	0	0.0379	0

Table 16. Specific activities of G441A and G441V mutants. The enzyme species were expressed in insect cells and the lysis supernatant was used as enzyme source. Product preparation and HPLC analysis were carried out as described in the methods section. The amount of 12R-LOX protein was determined by Western blot analysis. The specific activity is given in µg HETE formation/mg LOX protein during a 15 min incubation period.

In contrast, the G441V mutant was catalytically inactive. This result was quite surprising since Ala and Val are closely related with respect to their structures. They only differ by an additional CH3-group. Our observations indicate that this position is rather sensitive towards mutation and thus may be of relevance for the catalytic mechanism. SP-HPLC analysis of the reaction products indicated two major peaks, which co-migrated with authentic standards of 12- and 8-HETE (Fig. 21). As expected from previous experiments 12-HETE was the major oxygenation product for the wild-type enzyme but also for the G441A mutant. Interestingly, larger amounts of 8-HETE were formed by the mutant enzyme. Quantification of the HPLC traces revealed a 12-HETE/8-HETE-ratio of about 1.55 for the mutant enzyme (share of 8-HETE is 45 %). In contrast, the corresponding ratio for the wild-type enzyme was about 5.9 (share of 8-HETE 18 %). Thus, G441A mutation alters

the 12-HETE/8-HETE ratio of the murine 12(R)-LOX in favour of 8-HETE formation.

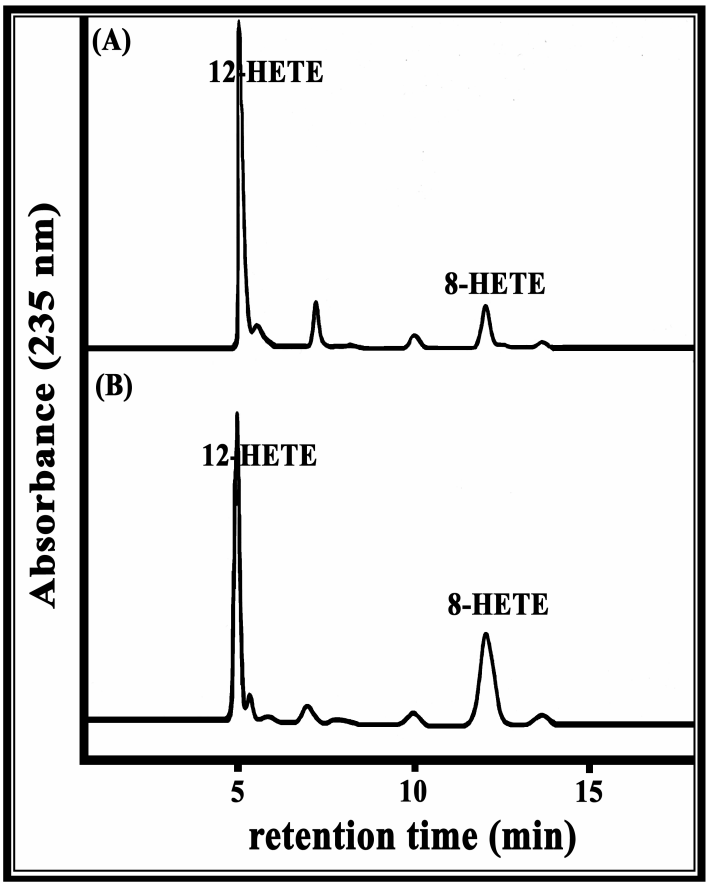


Fig. 21. Straight-phase HPLC chromatograms of wild type (A) and G441A (B) mutant with arachidonic acid methyl ester. Reaction products were purified by RP-HPLC and hydrolysed under alkaline conditions. After re-purification by RP-HPLC the free fatty acid derivatives were analysed by SP-HPLC on a Nucleosil 100-7 column. The solvent system used was *n*-hexane: Isopropanol (2%) with 0.1% acetic acid; flow rate 1 ml/min.

When we analysed the enantiomer composition of the two major reaction products (Fig. 22) by chiral phase HPLC we obtained the following results: i) As expected from previous experiments the 12-HETE formed by the wild-type 12(R)-LOX was predominantly the R-isomer. Only small amount of the corresponding S-enantiomer was found. In the same way, the R-isomer was dominating the 8-HETE peak. ii) Analysing the 12-HETE formed by the mutant enzyme species we observed an increase in the relative share of the S-

isomer when compared with the wild-type enzyme (R/S-ratio of 65:35 for the mutant vs. 92:8 for the wild type).

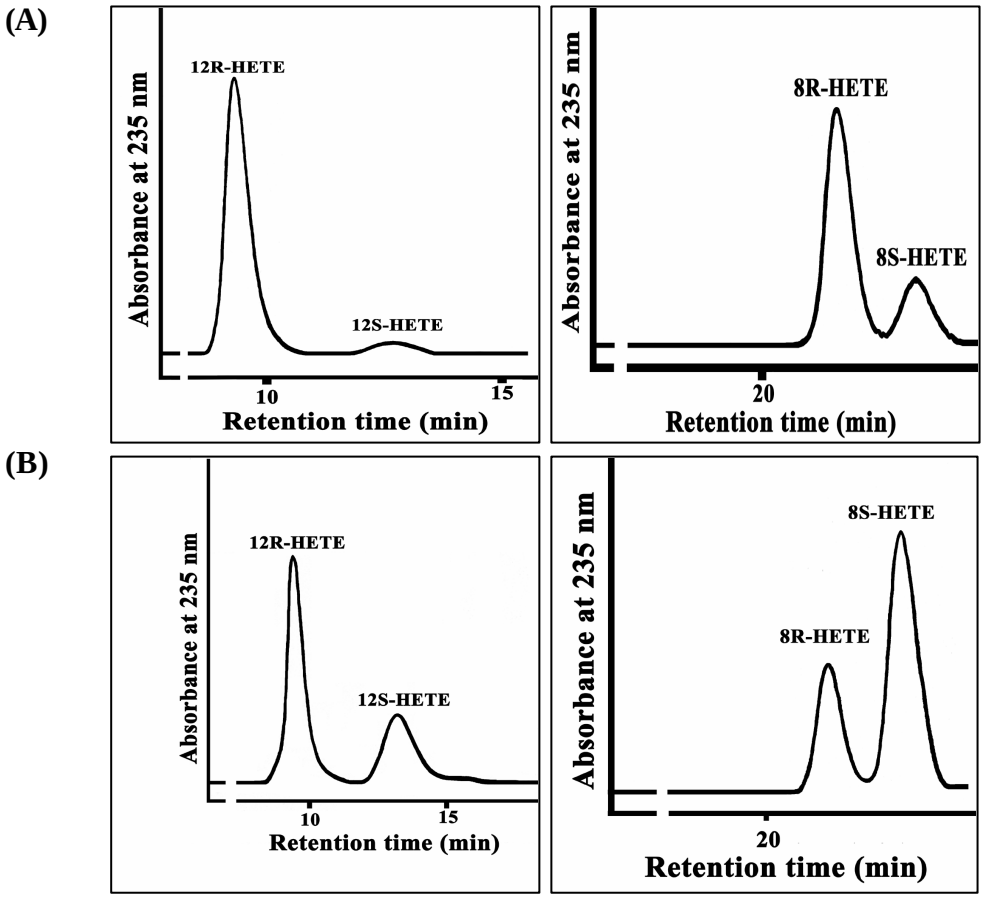


Fig. 22. Chiral-phase HPLC of wild type (A) and G441A (B) mutants. 12- and 8-HETE were purified by sequential RP- and SP-HPLC and further analyzed on a Chiralcel OB column using a solvent system *n*-hexane/isopropanol (2% for 12-HETE and 3% for 8-HETE) and 0.1% acetic acid; flow rate 1 ml/min.

More interestingly, however, were the results obtained during analysis of the 8-HETE formed by the mutant enzyme. Here we observed a reversal of the S/R ratio. With the wild-type enzyme 8R-HETE was dominant (R/S-ratio of 98:2) whereas 8S-HETE was the major isomer for the G441A mutant (R/S-ratio 30:70). The mechanistic reasons for this reversal of the S/R-ratio remain unclear. It may be speculated that an inverse orientation of the substrate molecule at the active site may contribute to this change.

In Table 17 the composition of the product patterns of arachidonic acid methyl ester oxygenation by the wild-type murine 12(R)-LOX and its G441A mutant are summarized. As indicated above the major outcome of these experiments was the reversal of the enantioselectivity of 8-HETE formation induced by G441A exchange.

Mutant	**Share (%)**			
	12-HETE	**11-HETE**	**9-HETE**	**8-HETE**
Wild Type	68% **(92R:8S)**	12%	6%	14% **(98R:2S)**
G441A	48% **(65R:35S)**	7%	7%	38% **(30R:70S)**

Table 17. Pattern of the oxygenation products formed from arachidonic acid methyl ester by the wild-type murine 12(R)-LOX and its G441A mutant. Reaction products were prepared by RP-HPLC and hydrolysed under alkaline conditions (see Methods section). After re-purification by RP-HPLC the free fatty acid derivatives were further analyzed by SP-HPLC and subsequently by CP-HPLC.

3.3.1.1. Reaction of the active mutants with linoleic acid methyl ester:

After having established the importance of G441 for the reaction specificity of arachidonic acid methyl ester oxygenation we carried out similar experiments using linoleic acid methyl ester as substrate. It has been reported before that the wild-type enzyme oxygenates linoleic acid methyl esters predominantly to 9R-HODE with 13S-HODE being a minor product (Siebert et al., 2001).

First we expressed the wild-type enzyme, the G441A and the G441V mutants in insect cells and determined their specific activities with linoleic acid methyl ester. In this system the wild-type enzyme and the G441A mutant exhibited an identical specific activity (Table 18). As expected from the results

of arachidonic acid methyl ester oxygenation the G441V mutant was enzymatically inactive. Thus, in principle there were no major differences in the specific activity assays using methyl arachidonate or methyl linoleate.

Mutant	**LOX activity (µg HODE/ml)**	**12R-LOX protein (mg/ml)**	**Specific Activity (µg HODE/mg protein)**
Wild Type	0.8	0.0335	24
G441A	0.8	0.0337	24
G441V	0	0.0379	0

Table 18. The specific activities of the G441A and G441V mutants of 12(R)-LOX with linoleic acid methyl ester expressed in the Sf9 insect cells. The recombinant enzymes were expressed in $15x10^6$ cells. The supernatants from lysed insect cells were incubated in PBS buffer with 100 µM linoleic acid methyl ester for 15 min at 37^0 C. The hydroperoxy derivatives of the fatty acids were reduced and then analysed on RP-HPLC. Activities were measured by comparison of the areas of the HODE and HODE methyl ester peaks with standards. The total protein used for the reaction is 0.15 mg/ml and the amount of 12R-LOX protein was calculated from the Western blot analysis. The specific activity is given in µg HODE formation/mg LOX protein during a 15 min incubation period.

When the pattern of the methyl linoleate oxygenation products were analysed we observed reversal of the positional specificity of the enzyme. As indicated in Fig. 23 and 24, 9(R)-HETE was the major product of linoleic acid methyl ester oxygenation. In contrast, the G441A mutant converted this substrate predominantly to 13(S)-HODE.

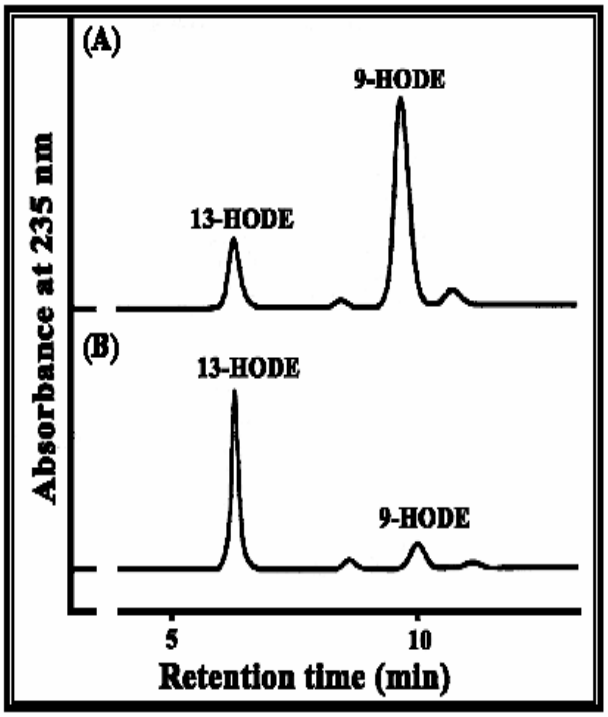


Fig. 23. Straight-phase HPLC of wild type (A) and G441A (B) mutant with linoleic acid methyl ester. Reaction products were purified by RP-HPLC and hydrolysed under alkaline conditions. After re-purification by RP-HPLC the free fatty acid derivatives were analyzed by SP-HPLC on a Nucleosil 100-7 column. The solvent system used was *n*-hexane: Isopropanol (2%) with 0.1% acetic acid; flow rate 1 ml/min

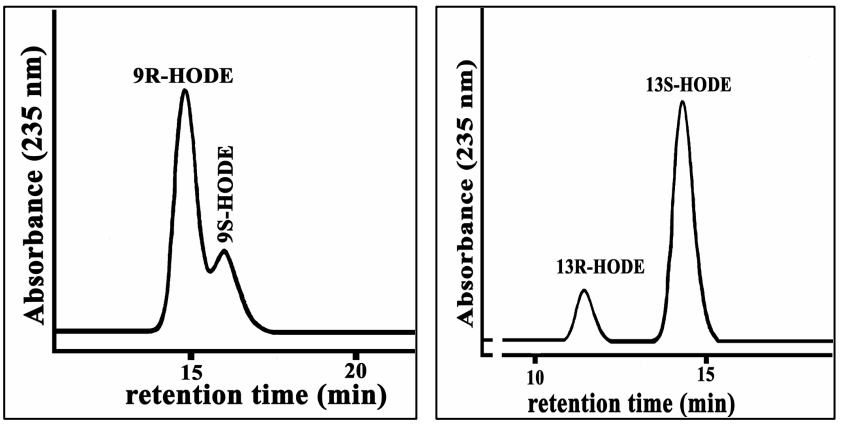


Fig. 24. Chiral-phase HPLC of the reaction products formed from linoleic acid methyl ester by G441A mutant. 9- and 13-HODE were isolated by SP-HPLC and further analyzed by CP-HPLC on a Chiralcel OB using a solvent system *n*-hexane/isoropanol (3%) and 0.1% acetic acid. The flow rate was 1 ml/min.

In Table 19 the results of the product analysis of linoleic acid methyl ester oxygenation are summarized. As indicated above the major outcome of this experiment is the reversal of the positional and optical specificity of the enzyme by G441A exchange.

Mutant	**Share (%)**	
	9-HODE	**13-HODE**
Wild Type	78 (92R:8S)	22 (18R:82S)
G441A	20 (74R:26S)	80 (15R:85S)

Table 19. Pattern of the oxygenation products formed from linoleic acid methyl ester by the wild-type murine 12R-LOX and its G441A mutant. Reaction products were prepared by RP-HPLC and hydrolysed under alkaline conditions (see Methods section). After re-purification by RP-HPLC the free fatty acid derivatives were further analyzed by SP-HPLC and subsequently by CP-HPLC.

SUMMARY OF THE RESULTS OF SECTION 3.3:

Multiple sequence alignments of various R-and S-LOX suggested that Gly441 of the murine 12R-LOX is conserved as Gly in all R-LOX. In contrast, this position is occupied by an Ala or a Ser in S-LOXs. We have modified Gly441 to Ala and Val by site-directed mutagenesis and observed following changes in specific activities and product pattern of the mutants.

- When we mutated Gly 441 of the murine 12R-LOX to a more space filling Ala (G441A) the positional specificity of the enzyme was found to be 50% of that of wild type enzyme while G441V was completely inactive both towards arachidonic acid methyl ester and linoleic acid methyl ester.

- G441A mutant resulted in 12-HETE:8-HETE (48:38) with the chirality of the products (12S/12R=20:80) and (8S/8R=80:20) in the mutant compared to the (12S/12R=2:98) and (8S/8R=20:80) of the wild type 12R-LOX.

- With linoleic acid methyl ester it formed more of 13-HODE than 9-HODE (80:20) opposite to that of wild type (20:80) with the chirality of the products (9S/9R=26:74) and (13S/13R=85:15) in the mutant compared to the (9S/9R=8:92) and (13S/13R=82:18) of the wild type.

3.4. DETERMINANTS OF SUBSTRATE SPECIFICITY

3.4.1. Chimeragenesis of human and mouse 12(R) - lipoxygenase:

The m12R-LOX and h12R-LOX share 86% homology but they differ completely in the substrate specificity. m12R-LOX prefers exclusively arachidonic acid methyl ester as its substrate while h12R-LOX reacts with free arachidonic acid. The differences may be due to the presence of three residues in the C-terminal region of the enzyme, which are quite different in human and mouse 12R-LOX.

Mouse 12(R)-LOX

E	G	F	A	**Q**	V	M	V	468
L	A	V	W	**Y**	A	M	E	510
S	G	F	P	**T**	C	L	R	558

Human 12(R)-LOX

E	G	F	A	**G**	V	M	V	468
L	A	V	W	**N**	A	L	E	510
S	G	F	P	**R**	C	L	R	558

Fig. 25. Sequence alignments of mouse and human 12(R)-LOX-species showing amino acid differences towards the C-terminal region of the enzyme .

In order to test the importance of these residues, we created a chimer that has human enzyme of about 500 bp containing the three residues

substituted in the same region of mouse lipoxygenase in the C-terminal region (see Methods section; 2.2.4)

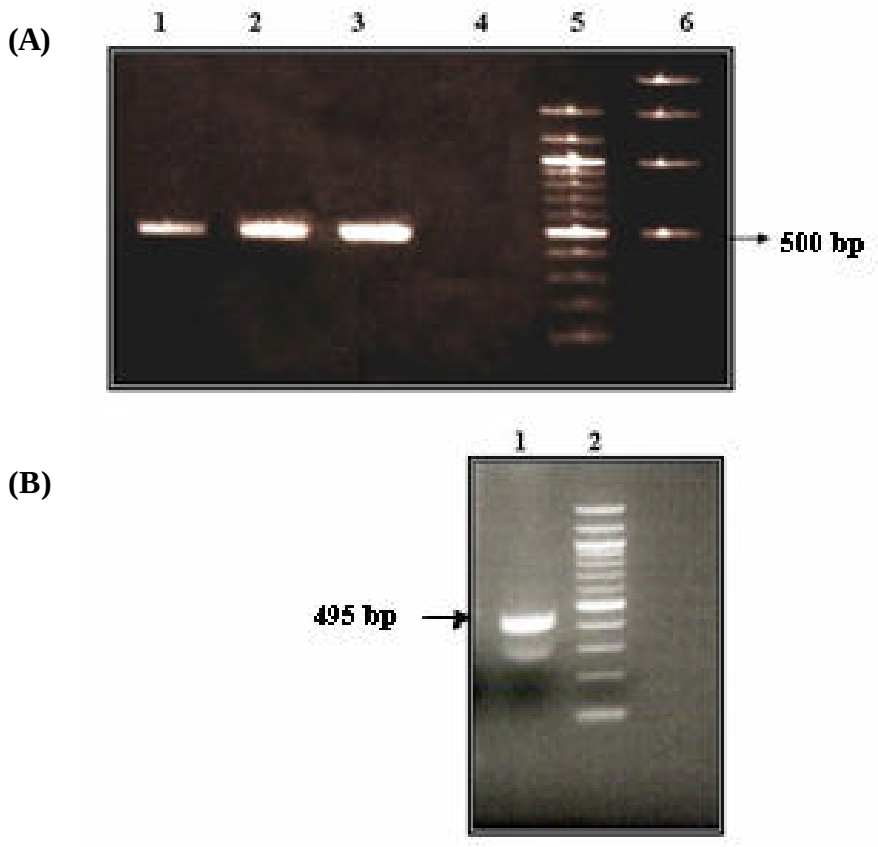


Fig 26. RT-PCR analysis of human 12(R)-lipoxygenase in human keratinocytes (HaCat cells). **Panel (A)** Lane 1,2,3: 500 bp region of human 12(R)-LOX. Lane 4: negative control (minus reverse transcriptase). Lane 5 and 6: Standards. **Panel (B)** Positive control (RT-PCR of GAPDH). Lane 1: 495 bp region and Lane 2: standard.

We could successfully express the chimeric enzyme in the insect cells having N-terminal the mouse 12R-LOX cDNA and the C-terminal human 12R cDNA. The chimera was expressed as a functional enzyme and the enzyme was reacted with the free arachidonic acid and also arachidonic acid methyl ester. It was found that the chimeric enzyme reacted well with both the substrates. With the arachidonic acid methyl ester it showed comparable activity as with the mouse enzyme and with the free arachidonic acid it showed about twice the activity (Table 20). This clearly indicates that the

human enzyme may contain amino acid determinants which are involved in the substrate specificity towards the charged arachidonic acid and the mouse enzyme lacks these determinants thereby changing the substrate specificity towards arachidonic methyl ester which is uncharged. The Table 21 shows the product pattern of the chimeric enzyme with the 12-HETE as the major product with the major R- stereoisomer.

Mutant	LOX activity (µg HETE/ml)	LOX protein (mg/ml)	Specific Activity (µg HETE/mg protein)
m12R-LOX with AA methyl ester	4.30	0.033	128
m12R-LOX with free AA	0.77	0.033	23
h+m chimer 12R-LOX with AA methyl ester	3.46	0.029	117
h+m chimer 12R-LOX with free AA	6.10	0.029	206

Table 20. The specific activities of the chimeric 12R-LOX (h+m) and mouse 12R-LOX (m 12R-LOX) expressed in the Sf9 insect cells. The enzyme species were expressed in insect cells and the lysis supernatant was used as enzyme source. Product preparation and HPLC analysis were carried out as described in the methods section. The amount of 12R-LOX protein was determined by Western blot analysis. The specific activity is given in µg HETE formation/mg LOX protein during a 15 min incubaton period.

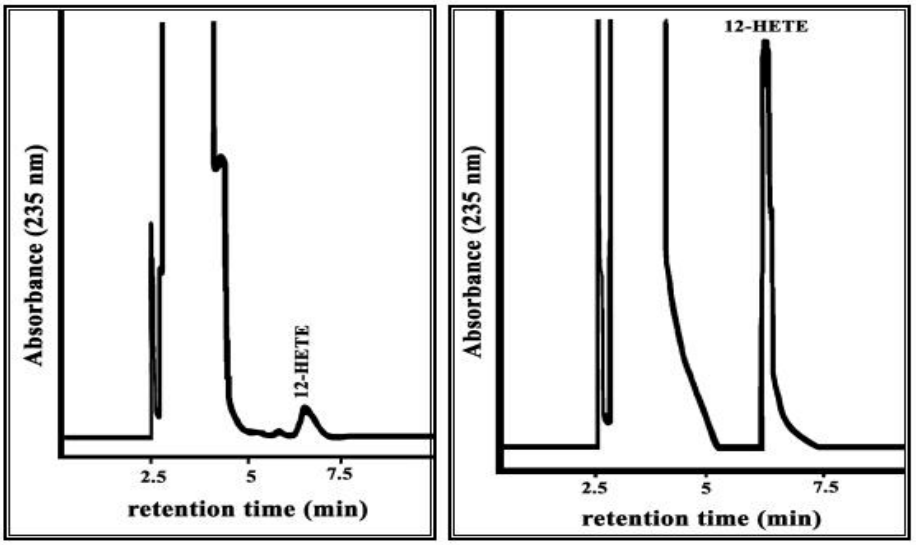


Fig.27. Reverse-phase HPLC chromatograms of wild type mouse 12R-LOX (A) and chimeric enzyme (mouse+human) 12R-LOX (B) with arachidonic acid: Reverse-phase analysis was done with lipid extracts from the assays of wild type enzyme and chimeric enzyme with arachidonic acid. The column used was Nucleosil 100-7 column (250x4 mm, Machery-Nagel, Düren, Germany) with SPD-M6A-Detector (Shimadzu). The detection of the products was at 235 nm. The solvent system used was methanol : water with 0.1% acetic acid. The flow rate was 1 ml/min. Authentic standards were used as external standards.

Mutant	**Share (%)**			
	12-HETE	**11-HETE**	**9-HETE**	**8-HETE**
m12RLOX+ AAMeEster	68% **(92R:8S)**	12%	6%	14% **(98R:2S)**
h+m12RLOX +AAMeEster	68% **(95R:5S)**	14%	3%	15% **(80R:20S)**
h+m12RLOX +Free AA	73% **(90R:10S)**	14%	7%	5% **(76R:24S)**

Table 21. Pattern of the oxygenation products formed from arachidonic acid methyl ester and free arachidonic acid by the wild-type murine 12R-LOX and the chimeric (human+mouse) 12R-LOX. Reaction products were prepared by RP-HPLC and hydrolysed under alkaline conditions (see Methods section). After re-purification by RP-HPLC the free fatty acid derivatives were further analyzed by SP-HPLC and subsequently by CP-HPLC.

SUMMARY OF THE RESULTS OF SECTION 3.4:

The human 12R-LOX and the mouse 12R-LOX show different substrate specificities indicating a probable involvement of substrate determinants which determine the substrate specificity. The two enzymes are 86% similar and major amino acids differences were concentrated towards the C-terminal region of the enzyme. A chimeric enzyme was created to test whether these amino acids are important for the substrate specificity.

- The (h+m) 12R-LOX gene cloned in pVL 1392 was expressed in insect cells as a functional protein and showed activity both towards free arachidonic acid and its methyl ester.

- The chimer enzyme showed more activity towards the free arachidonic acid (206 µg HETE/mg LOX protein) than towards its methyl ester (117 µg HETE/mg LOX protein).

- The major product of the chimer was 12R-HETE with both the substrates indicating that the change in the gene sequence resulted only in the change in substrate specificity. It proves that the determinants responsible for the substrate specificity reside in the C-terminal region of the 12R-LOX.

3.5. IRON LIGANDS – THE IMPORTANCE OF THE FIFTH IRON LIGAND

3.5.1. Asparagine582:

In view of the central role of the iron in lipoxygenase catalysis, amino acid residues as possible iron ligands were extensively investigated by site-directed mutagenesis. A sequence of His- $(X)_4$-His- $(X)_4$-His- $(X)_{17}$-His- (X)

$_{8}$-His in soybean lipoxygenases was suggested as an iron-binding region (Shibata, 1988). This cluster, together with some other histidine residues (His-131, -433 and –551 in human 5-lipoxygenase) is highly conserved in various mammalian lipoxygenases. Later, the crystallographic data showed that COO- of the C-terminal isoleucine was another ligand (Boyington et al., 1993). The C-terminal isoleucine is well conserved in mammalian lipoxygenases and its deletion in human 5-lipoxygenase results in the loss of enzyme activity and also of iron (Hammarberg et al., 1995). While all the crystal structures show four ligands in common, one of the structures of the soybean lipoxygenase L-1 and the rabbit reticulocyte 15-LOX structure have a fifth amino acid ligated to the iron (Minor, 1996 ; Gillmor, 1997). The importance of this fifth ligand is not clearly known in lipoxygenase structure–function. In support of its potential significance, mutation of this residue to alanine or valine by site-directed mutagenesis in soybean L-3 resulted in a complete loss of catalytic activity (Kramer et al., 1994). In the human 5-lipoxygenase, mutation of the corresponding asparagine to aspartate or alanine reduced activity to about 1% that of wild type, while replacement with glutamine eliminated enzymatic activity; these mutations did not affect the iron content of the enzyme (Hammerberg et al., 1995). This putative fifth iron ligand of lipoxygenases is conserved as Asn or His (Shibata et al., 1987, Sigal et. al., 1988 and Funk et al., 1996), except within a family of mammalian lipoxygenases represented by the second type of 15-lipoxygenase (e.g., human 15-LOX-2) and the phorbol-ester-inducible mouse 8-LOX where these enzymes have a serine residue in the position equivalent to Asp-694 of the soybean enzyme or His-545 of the reticulocyte-type of 15-LOX (Brash et al., 1997; Jisaka at al., 1997; Krieg et al., 1998; Liminga and Oliw, 1999). An interesting change in positional specificity of the 8-LOX enzyme was observed in the Asn and His mutants. These substitutions induced a change from pure 8-LOX specificity in the wild-type enzyme to one that forms some 15S-HETE (Jisaka et al., 2001).

Mouse 8(S)-LOX								
H	A	A	V	**S**	S	G	Q	F
562								
Mouse 12(R)-LOX								
H	A	A	V	**N**	S	G	Q	L
586								
Mouse 5(S)-LOX								
H	A	A	V	**N**	F	G	Q	Y
559								
Rabbit 15(S)-LOX								
H	S	S	I	**H**	L	G	Q	L
459								

Fig. 28. Sequence alignments of various LOX-species surrounding the putative fifth iron ligand.

From Fig. 28, Asparigine582 was identified as fifth iron ligand in 12(R)-lipoxygenase and to investigate the importance of this residue in murine 12R-LOX structure-function and see if there is a change in positional specificity, the Asn was replaced by Serine and Histidine by site-directed mutagenesis and expressed in the insect cells. The specific activities and the product profiles of the mutant species were analysed. From the Table 22 it is seen that there is no drastic alterations in the specific activities of the enzyme species compared to the wild type enzyme. Here we show that changing the Asn582 to Ser and His modifies but does not greatly compromise the catalytic activity of the enzyme. The N582S mutant was active retaining about 50% of the activity of the wild type while the N582H showed comparable amount of activity with the wild type. The main HETE product of Ser mutant was 12-HETE as determined by SP-HPLC analysis (Fig. 29) and its chirality was confirmed to be R-isomer by chiral phase HPLC (data not shown). An interesting change in positional specificity of the enzyme was observed in His mutant with 11-HETE as the major product (Fig. 29) and it was found to be predominantly R when analysed on CP-HPLC (Fig. 30). The inference is that

the change in sequence leads to small changes in the tertiary structureof the protein leading to altered positional specificity.

Mutant	LOX activity (µg HETE/ml)	12R-LOX protein (mg/ml)	Specific Activity (µg HETE/mg protein)
Wild Type	2.99	0.072	41
N582S	2.07	0.087	24
N582H	2.70	0.072	37

Table 22. The specific activities of the N582S and N582H mutants of 12R-LOX expressed in the Sf9 insect cells. The recombinant enzymes were expressed in $15x10^6$ cells. The supernatants from lysed insect cells were incubated in PBS buffer with 100 µM arachidonic acid methyl ester for 15 min at 37^0 C. The hydroperoxy derivatives of the fatty acids were reduced and then analysed on RP-HPLC. Activities were measured by comparison of the areas of the HETE and HETE methyl ester peaks with standards. The total protein used for the reaction is 0.15 mg/ml and the amount of 12R-LOX protein was calculated from the Western blot analysis. The specific activity is given in µg HETE formation/ mg LOX protein during a 15 min incubation period.

Mutant	Share (%)			
	12-HETE	**11-HETE**	**9-HETE**	**8-HETE**
Wild Type	63% **(96R:4S)**	10%	8%	19% **(78R:22S)**
N582S	69% **(97R:3S)**	10%	6%	15% **(81R:19S)**
N582H	22% **(90R:10S)**	60% **(96R:4S)**	8%	10% **(75R:25S)**

Table 23. Straight-phase and Chiral-phase analysis of the reaction products of N582S and N582H mutants of 12(R)-lipoxygenase with arachidonic acid methyl ester as substrate. Straight phase analysis was done with HETE fractions (HETE

methyl ester fraction was hydrolysed) of RP-HPLC. The different products separated on SP-HPLC were further analysed on CP-HPLC. Authentic standards were used as external standards. The concentrations of different products and S and R enantiomers were determined from the peak areas and are used to determine the ratios, the sum of which was set to 100%.

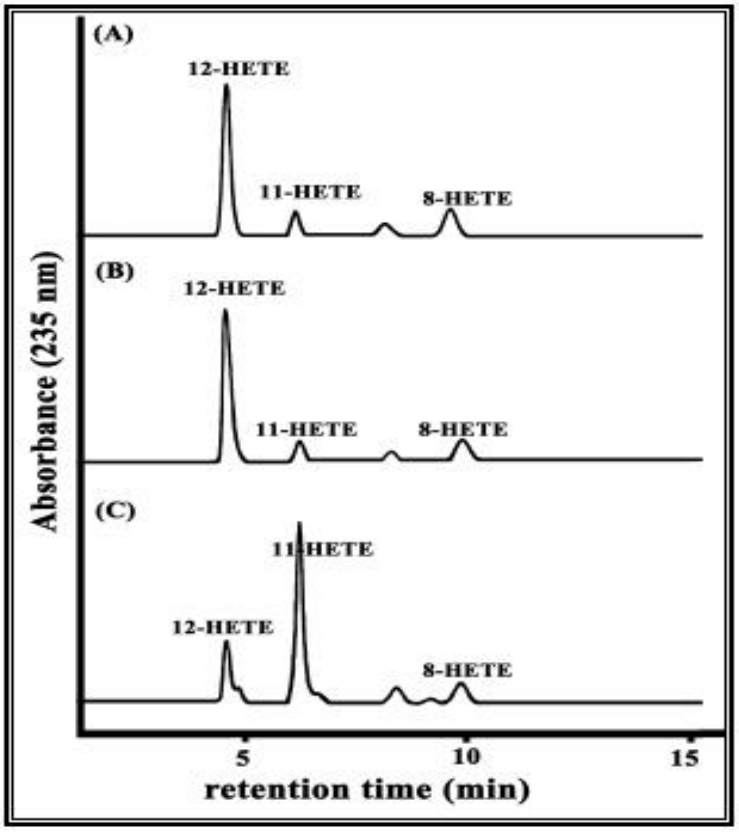


Fig. 29. Straight-phase HPLC chromatograms of wild type (A) N582S (B) and N582H (C) mutants with arachidonic acid methyl ester: Straight- phase analysis was done with HETE fractions (HETE methyl ester fraction was hydrolysed) obtained from RP-HPLC column (as described in the Material and Methods section).

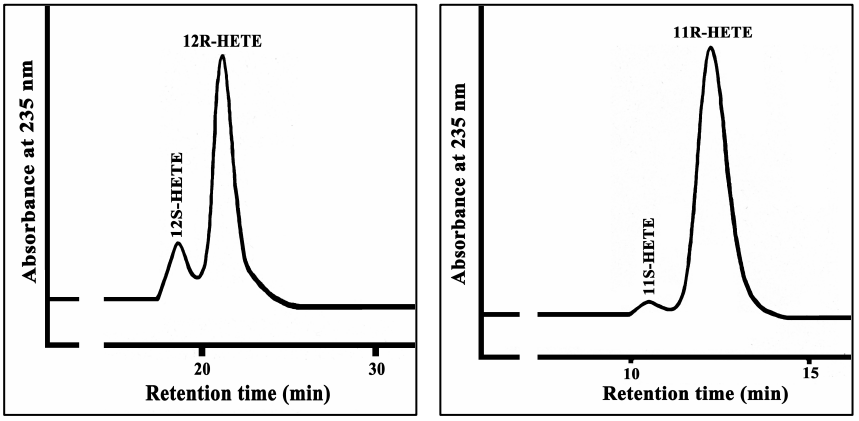


Fig. 30. Chiral-phase HPLC chromatograms of N582H mutant: Chiral-phase analysis of 12- and 11-HETEs formed by the N582H mutant. The 12- and 11-HETE eluates from SP-HPLC were subjected to chiral phase chromatography on a Chiralcel column, 250x4.6 mm, (Diacel Chem. Industries, USA) with solvent system *n*-Hexan/2-Propanol (2%) and 0.1% acetic acid. The flow rate was 1 ml/min.

SUMMARY OF THE RESULTS OF THE SECTION 3.5:

The fifth putative iron ligand was found to be important for some but not to all the lipoxygenases. We have carried out mutations of this ligand in 12R-LOX to see if the change affected the structure-function of the enzyme. The putative fifth iron ligand was replaced by serine and histidine by site-directed mutagenesis and expressed in the insect cells.

- The mutational changes did not result in a drastic change in the enzyme activities of the enzyme species. The His mutant showed a similar level of lipoxygenase activity (37 µg/mg protein) to that of wild type (41 µg/mg protein) whereas the Ser mutant had lesser activity (24 µg HETE/mg LOX protein).

- There is no change in the product pattern of N582S mutant which showed 12R-HETE as the major product but the Asn to His substitution resulted in a shift of 12R-HETE (22%) towards 11R-HETE (60%). This indicated that the substitution at this position with histidine leads to small changes in the tertiary structure of the protein affecting the proper alignment of the substrate at the active site of the enzyme.

3.6. EXTRA DOMAIN IN R-LIPOXYGENASES

3.6.1. Deletion mutation studies (changes to the structure of the lipoxygenase enzyme):

With 701 amino acid residues 12(R)-lipoxygenase is unique among all mammalian lipoxygenases. The larger size has been attributed to an additional

proline-rich stretch of 31 amino acids inserted between P154 and P185 which does not corresponds to any other known plant or mammalian lipoxygenase sequences.

We made deletion mutations of the stretch and observed the specific activities of the enzyme species. We did three types of deletion studies. We have removed the entire stretch (P154-P185), the first five amino acids of the stretch (P154-I159) and the last five amino acid of the extra stretch (Y180-P185).

Mutant	LOX activity (µg HETE/ml)	LOX protein (mg/ml)	Specific Activity (µg HETE/mg protein)
Wild type	0.40	0.33	1.20
Del 154P-185P	0.090	0.14	0.61
Del P154-I159	0.093	0.37	0.25
Del Y180-P185	0.28	0.34	0.82

Table 24. Quantification of the specific activities of deletion mutants of the murine 12R-LOX expressed in bacterial cells using arachidonic acid methyl ester as substrate. The mutants were created as described in the Methods section and bacterial lysates were used as enzyme source. LOX activity was determined by HPLC quantification of the reaction products. The amount of LOX protein in the lysates was quantified by Western blotting using the pure his-tag rabbit 15-LOX as mass standard.

Deletion studies of the extra 31 amino acids resulted in:

1. Removal of the entire stretch (from P154 to P185) resulted in about 50% loss of the specific activity.

2. Removal of the first 5 amino acids in the N-terminal region (P154-I159) resulted in 80% loss of the activity and
3. Removal of the last 5 amino acids at the C-terminal region (Y180-185) showed no major (only about 30%) loss of the activity.

The products formed in the first two deletion studies were unspecific whereas the removal of last 5 amino acids from the C-terminal region resulted in the product profile similar to wild type indicating no major change in the enzyme conformation (data not shown).

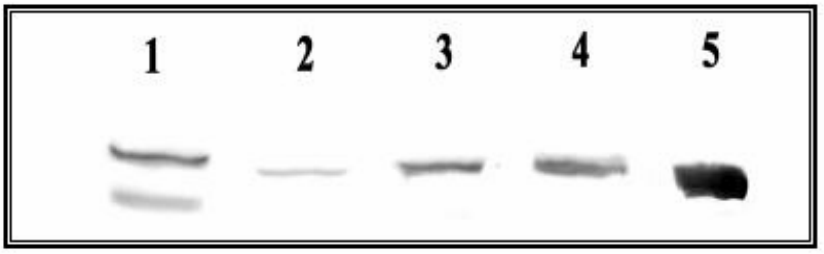


Fig. 31. Western blot analysis of deletion mutations of murine 12(R)-lipoxygenase expressed in bacterial cells. Bacterial cells were transformed with the cDNA of the mutants and the protein expressed. The cells were then lysed and the clear lysates (50 µg protein) electrophorized on 12% SDS-PAGE. The proteins were electroblotted onto a nitrocellulose membrane and incubated with anti-histidine antibodies. Protein was detected by an enhanced chemiluminscence method (described in materials and methods). Lane 1: wild type, Lane 2: P154-P185 deletion mutant, Lane 3: P154-I159 deletion mutant, Lane 4: Y180-P185 deletion mutant, Lane 5: known concentration (1 µg) of rabbit reticulocyte 15(S)-lipoxygenase used as standard.

From the Western blot analysis of the deletion mutations (Fig. 31), the P154-185 deletion mutant showed a fainter band compared to the wild type and other mutants. Loss of 31 amino acids may involve a complete alteration of the protein conformation leading to the altered enzyme activity and protein expression. Interestingly, the P154-I159 deletion mutant showed very low level of activity, lower than the P154-P184 mutation indicating that the first five amino acids may be responsible for the proper folding of the enzyme for optimum activity. The protein expression level of P154-159 as seen from the western blot analysis showed comparable level of expression as the wild type.

The deletion of the last five amino acids of the stretch Y180-P185 did not show any drastic change in the specific activity and the product pattern of this mutant was similar to the wild type enzyme with 12R-HETE as the major product (data not shown).

DISCUSSIOn

4.1. MECHANISM OF 12(R)-LIPOXYGENATION

Lipoxygenases (LOXs) are non-heme iron containing dioxygenases that catalyze the regioselective and enantioselective oxidation of unsaturated fatty acids containing one or more (Z, Z)-1,4-pentadienoic moieties. The stereochemistry of the dioxygenation process is tightly controlled and has several characteristic features: (i) one of the pro-chiral H-atoms (pro-S or pro-R) is stereospecifically removed from the central bisallylic methylene group of a pentadiene system of the substrate. (ii) Assuming a planar structure of the pentadienoic system oxygen insertion and hydrogen abstraction proceed antarafacially (iii) E,Z-conjugation of the double bonds of the pentadiene system leading to chromophore with a strong UV-absorbance at 234 nm. Fatty acid hydroperoxides formed are further metabolized in multiple ways in different organisms, leading to leukotrienes and related products in mammals, and to a variety of oxylipins in plants and fungi (Hamberg and Gerwick 1993; Gardner 1991; Samulesson and Funk 1989).

Until the mid-1990s, all the cloned lipoxygenases were from plant or mammalian sources, and all exhibited, S-stereospecificity. The first lipoxygenase to be characterized with pure R-stereospecificity was an 8R-LOX that was purified and subsequently cloned from the prostaglandin containing Caribbean coral *Plexaura homomalla* (Brash et al., 1996). From the cDNA of this coral, a different 8R-LOX was also cloned, one that occurred as part of a natural fusion protein, which consisted of an N-terminal catalase-related allene oxide synthase domain and a C-terminal lipoxygenase domain (Koljak et al., 1997). This second 8R-LOX was closer in sequence to mammalian S-lipoxygenases (35–40% amino acid identity). Later on, a 12R-LOX was discovered in human skin, which has been implicated in the biosynthesis of 12(R)-HETE in this organ (Boeglin et al., 1998). At the same time the 12R-LOX gene was characterized in the mouse skin (Krieg et al., 1999). Thus, a novel class of R-specific–LOX was described for the first time in the animal kingdom. Based on amino acid sequence alignments, R-LOXs

share a high degree of structural homology with their S-specific counterparts and consist of a small N-terminal beta-barrel domain and a larger catalytic domain. The S-specific, human 15-LOX-2 shares about 50% amino acid sequence identity with the human 12R-LOX. This indicates a closer relatedness between these two enzymes when compared with that of human 15-LOX-1 and human 15-LOX-2 (35–40%). The close relatedness between R- and S-lipoxygenases indicate that both groups of enzymes belong to the same family. There seem to be no obvious single amino acid determinants for the opposite stereo specificity. While details on the basis of the stereospecificity remain to be determined, an important principle was established. The opposite stereospecificity is most likely related to the substrate orientation at the active site and to the stereochemical control mechanisms, which appear to be different for the two enzyme subfamilies. To rationalize the differences in substrate orientation between R- and S-LOX a topological model was proposed that involves a turn around of the fatty acid at the active site, such that either the carboxyl end or the omega carbon chain is in equivalent positions in different enzymes. In addition, the position of oxygen insertion into the pentadienyl radical formed during initial hydrogen removal is different between R- and S-LOXs. Thus, at least two elementary reactions impacting the stereochemistry of the fatty acid oxygenation are different between the two LOX subfamilies. If this model is applied to the oxygenation of arachidonic acid a close relatedness between 8R- and 12S-oxygenation becomes apparent. Both reactions involve an initial removal of the pro-S hydrogen from C-10. If oxygen insertion proceeds antarafacially either 8R- or 12S-HpETE is formed depending on the direction of radical rearrangement.

Thus, formation of 8S- and 12R-HPETE follows similar stereochemical control principles except from substrate binding in an inverse head to tail orientation. The concept that fatty acid substrates can bind one way or other at the active site of LOXs is well documented in the lipoxygenase literature (van Os et al., 1981; Ivanov et al., 2001). Although there is no direct information on the structure of productive LOX-substrate complexes for any

LOX-isoform the above explained model is plausible and explains satisfactorily most experimental data available so far.

4.2. RECOMBINANT EXPRESSION OF THE MURINE 12(R)-LOX AND ENZYME CHARACTERIZATION:

To test this theory in more detail we used murine epidermal 12R-LOX lipoxygenase as a model system and mutated those amino acid residues, which have previously been shown to be important for the stereochemistry of other LOX-isoforms. This thesis discusses the primary determinants of the reaction specificity of murine 12R-LOX, which includes the determinants for the positional specificity, the stereoselectivity and the substrate specificity.

Mammalian skin is a tissue with extensive fatty acid metabolism and various LOX-isoforms contribute to fatty acid turnover. Four lipoxygenase isoenzymes have been characterized and cloned from this source, including a 15-lipoxygenase-2 from human skin (Brash et al., 1997), an epidermis type 12-lipoxygenase in both human and mouse (van Dijk et al., 1995; Kinzig et al., 1997; Sun et al., 1998), a phorbal ester inducible 8-lipoxygenase from mouse (Jisaka et al., 1997; Krieg et al., 1998) and 12R-LOX from human and mouse skin (Boeglin et al., 1998; Krieg at al., 1999). With 701 amino acid residues the latter enzyme is unique among all mammalian lipoxygenases. The large size has been attributed to an additional proline-rich stretch of 31 amino acids, which is not found in any other plant or mammalian lipoxygenase sequences. The human 12R-LOX was found to metabolize arachidonic acid almost exclusively into 12R-HETE. The murine orthologue protein is inactive with free arachidonic acid and free linoleic acid but strongly prefers the corresponding methyl esters. This surprising substrate specificity of mouse 12(R)-lipoxygenase might indicate that esterified rather than free fatty acids may represent the natural substrates for this enzyme. However, such

arachidonyl esters do not include arachidonic acid containing phospholipids since these lipids are not oxygenated at all by the enzyme (Krieg et al., 1999).

To study the murine epidermal 12R-lipoxygenase the enzyme was first overexpressed as his-tagged fusion protein in *E.coli* and later in the baculovirus/insect cell expression system. Although there was successful expression of the enzyme in both systems (in *E.coli* in a yield of 1 mg/L culture fluid and in the baculovirus/insect cell system in a yield of 0.1 mg/15x10^6 cells) its specific activity was low when compared with the recombinant reticulocyte 15S-lipoxygenase expressed under similar conditions. However, the enzyme exhibited a similar catalytic activity similar as other lipoxygenases of epidermal origin such as epidermis type 12(S)-lipoxygenase and 8-lipoxygenase (Kinzig et al., 1997; Jisaka et al., 1997; Krieg et al., 1998). There are several options to explain these differences in the catalytic efficiencies between the classical S-LOXs (5S-LOXs, 15-LOX1) and some epidermal LOX-isoforms such as the murine 12R-LOX. One possibility is that the physiological substrates of this particular isoform have not yet been identified. Moreover, keratinocyte-specific post-translational modifications of the enzymes such as phosphorylation, glycosylation and non-covalent interactions with activating proteins may be involved. Interestingly, in our hands the specific activity of the enzyme expressed in the baculovirus/insect cells was about 4.5 times higher when compared with the enzyme of bacterial origin. These data suggest the importance of post-translational modification for the catalytic activity.

HPLC analysis of the products formed from arachidonic acid methyl ester by the recombinant enzyme showed two major peaks, which cochromatographed with authentic standards of free and methylated 12-HETE. Since free arachidonic acid is not metabolized by the enzyme we concluded that the free 12-HETE constitutes an hydrolysis product of 12-HETE methyl ester formed by the 12R-LOX. In fact, bacterial lysate and homogenates of insect cells contain unspecific lypolytic activities capable of hydrolyzing fatty acid methyl esters. Arachidonic acid esters are also accepted as substrates by

the reticulocyte 15-lipoxygenase and leukocyte type 12-lipoxygenase (Takahashi et al., 1993; Kühn et al., 1994). Nevertheless, the latter enzymes preferentially oxygenate the free acid. When the methyl ester peak was further analysed on the SP-HPLC, 12-HETE methyl ester was the major peak, but smaller amounts of 11-HETE?methyl ester and 8-HETE methyl ester were also formed. With linoleic acid methyl ester as the substrate the major product was 9R-HODE methyl ester.

Enzyme purification was performed using the Ni-Agarose affinity column but only about 50% protein bound to the column and also the protein was found to be extremely labile during purification process. Several attempts were made to optimise the purification procedure using several modifications like changes in the pH of the binding and elution buffers and purification under denaturing conditions using 6 M urea. These changes did not result in the increase in purification of the enzyme. This indicates that the murine epidermal 12R-lipoxygenase might require either specific factors or associations with other proteins from a specific epidermal cell type to yield fully active, functional protein.

4.3. DETERMINANTS FOR POSITIONAL SPECIFICITY:

The regiospecificity of the lipoxygenase product formation depends on the positional specificity of both initial hydrogen abstraction and subsequent dioxygen insertion. Detailed insights into the regiospecificity of the lipoxygenase-catalyzed dioxygenation reaction were obtained from site-specific mutagenesis experiments. Experiments on mammalian 12-and 15-LOXs indicated that the site of hydrogen abstraction could be altered when critical amino acids are targeted by site-directed mutagenesis. When the space-filling I418, F353 and I593 of the human and/or rabbit reticulocyte-type 15-LOX were mutated to smaller residues, the substrate fatty acids slide farther into the substrate-binding pocket approaching the doubly allylic carbon-10 of arachidonic acid, closer to the catalytically active non-heme iron, leading to

the hydrogen abstraction at that carbon atom and insertion of molecular oxygen at C-12 forming 12-HETE. Based on these mutagenesis studies two hypothetical models were put forward to explain the substrate alignment at the active site: (i) space-based model and (ii) orientation model (Kühn, 2002).

Sequence alignments between the rabbit 15-LOX and the mouse 12R-LOX identified the corresponding amino acids in 12R-LOX, (A455, F390 and V631) and these residues constituted suitable targets for site directed mutagenesis. First, these amino acids were mutated to residues with more bulky side chains and the reaction products of arachidonic acid methyl ester oxygenation by these mutants were analysed. The results indicated that this strategy did not result in major alterations in the positional specificity of the enzyme suggesting that the structural basis of the positional specificity of 12R- LOXs appear to be different from that of 15-LOX-1. Since there is no direct structural information on the active site of the murine 12R-LOX it remains unclear whether or not our mutagenesis strategy did really lead to an increase in the volume of the active site. It might well be said that the active sites of the two enzymes are differently structured so that the mutations carried out have no dramatical impact on the volume of the 12R-LOX active site. Alternatively, one might assume that substrate alignment at the active site for this LOX-isoform was different from that of 15-LOX-1.

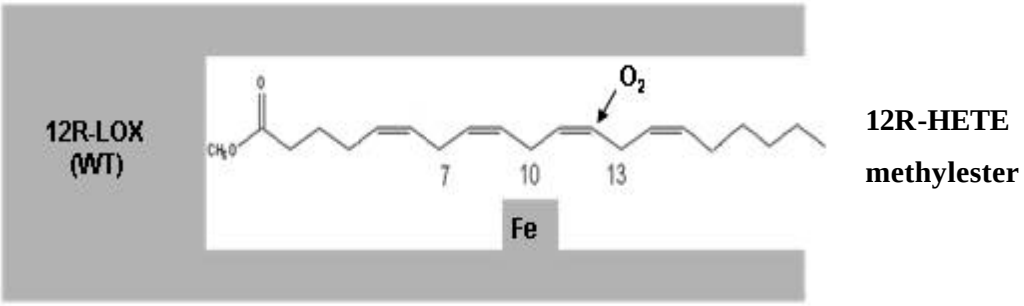
Next, we mutated the medium-sized amino acid V631, which corresponds to I593 of the rabbit 15-LOX, to smaller residues (Alanine and Glycine). Here we observed a dramatic change in the product pattern. The major oxygenation product of the wild-type enzyme was 12R-HETE but that of the mutants was 11R-HETE. These results indicate prominent changes in the reaction mechanism since both, the site of hydrogen abstraction and the direction of radical rearrangement were altered. When a chemically modified substrate such as 20-HETE methyl ester was used as substrate, the major product formed in the wild type enzyme was 8, 20-diHETE, while in the mutants it was 9, 20-diHETE.

One way to explain this mechanistic difference is to assume an inverse substrate alignment at the active site. In this case the substrate fatty acid slides into the substrate-binding pocket with the carboxylate end ahead. Burying a strongly polar free carboxylate inside the hydrophobic environment of the substrate-binding pocket may be thermodynamically hindered and thus, free fatty acids are not accepted as substrate for this LOX isoform. However, methylation reduces the polarity of the carboxylate and thus, may decrease thermodynamic hindrance. A similar situation was previously reported for the oxygenation of 15-HETE by S-LOXs from rabbit reticulocytes and soybeans (van Os et al., 1981; Schwarz et al., 1998). Both enzymes catalyze major 5(S)- and 8(S)-lipoxygenation of 15-HETE, which appears to involve an inverse substrate with the free carboxylate being buried deeply inside the hydrophobic substrate-binding pocket. The thermodynamic hindrance of such an enzyme substrate complex is mirrored by the low substrate affinity and the strongly impaired reaction rate. Here again, methylation of the carboxylate strongly increased both substrate affinity and reaction rate (Schwarz et al., 1998).

Formation of 12(R)-HETE involves C10-hydrogen abstraction and [+2]-radical rearrangement. In contrast, synthesis of 11(R)-HETE requires C13-hydrogen abstraction and [-2]-radical rearrangement. Thus, molecular dioxygen was inserted form the same side of the plane determined by double bonds. The structural reasons for these mechanistic differences appear to be rather complex and cannot be explained solely on the basis of the space-related hypothesis of the positional specificity. Assuming that V631 is an active site amino acid, introduction of Ala or Gly at this position increases the volume of the substrate-binding site allowing arachidonic acid methyl ester to slide deeper into the pocket. If the substrate is inversely aligned at the active site (carboxylate end slides in first) as suggested for the wild type enzyme such deeper penetration enables hydrogen removal from C13 instead from C10. Thus, for the wild type enzyme and for the mutants arachidonic acid methyl ester appears to be similarly oriented at the active site of the enzyme

(Fig. 32). The mechanistic reasons for the inverse directions of radical rearrangement remain unclear.

(A)



(B)

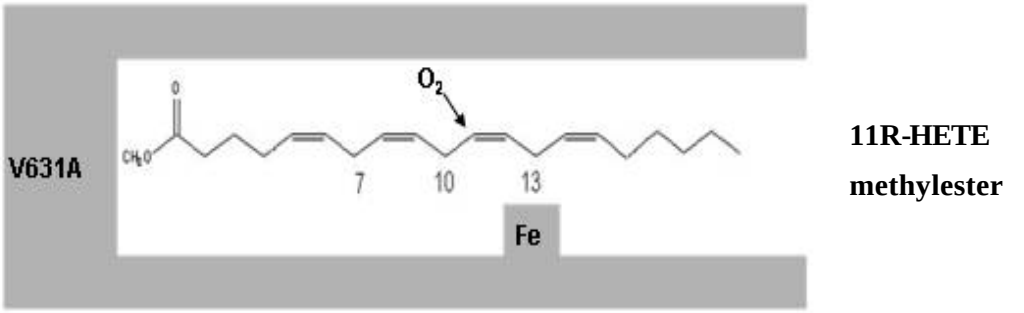


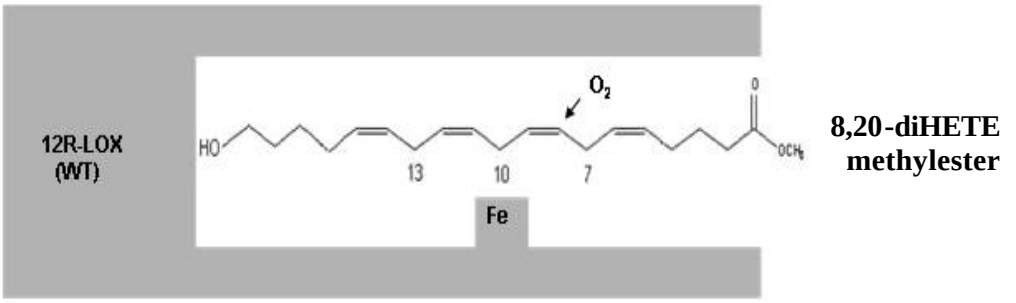
Fig. 32. Schematic models of substrate alignment at the active site of the murine 12R-LOX: It is shown here that the arachidonic methyl ester enters the active site with its carboxylate end ahead. Panel A: Wild type 12R-LOX which gives 12R-HETE as its major product ans Panel B: V631A mutant which gives 11R-HETE as the major product.

A similar situation appears to be the case when 20-HETE was used as substrate. Free 20-HETE was not a good substrate for the murine 12(R)-LOX but its methyl ester was well oxygenated. These data suggest that 20-HETE methyl ester may slide into the substrate-binding pocket with its methyl end ahead. The major product of 20-HETE methyl ester oxygenation by the wild type enzyme was 8, 20-diHETE and formation of this compound involves C10-hydrogen removal and [-2]-radical rearrangement. In contrast, the main product of 20-HETE methyl ester oxygenation by the V631A mutant was 9, 20-diHETE. Formation of this compound involves C7 hydrogen removal and [+2]-radical rearrangement. These results can be explained if the substrate

enters the active site with its omega hydroxyl end first and by a deeper penetration of the substrate into the substrate-binding pocket.

Linoleic acid contains only one bisallylic methylene and thus the pattern of theoretically possible reaction products is more simple. Murine 12R-LOX converted linoleic acid methyl ester mainly to 9(R)-HODE and to about 10% of 13-HODE. The chiral phase HPLC indicated the strong preponderance of the S-enantiomer for the 13-HODE. These data indicate that the stereochemistry of 9(R)-HODE and 13(S)-HODE formation is completely controlled by the enzyme. In contrast to arachidonic acid methyl ester the specificity of methyl linoleate oxygenation was not altered by V631A exchange.

(A)



(B)

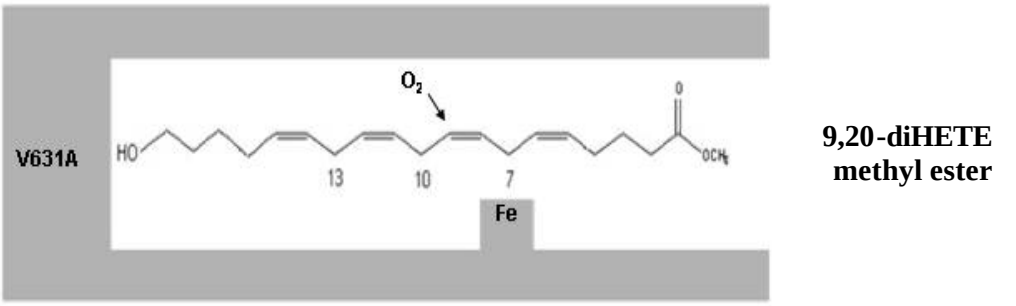


Fig. 33. Schematic representation of substrate alignment at the active site of murine 12R-LOX with 20-HETE methyl ester as the substrate. Panel A. Wild type enzyme which forms 8, 20-diHETE as the major product with the substrate. **Panel B**. V631A mutant which gives 9,20-diHETE as the major product.

4.4. DETERMINANTS FOR STEREOSPECIFICITY:

The next set of experiments were designed to identify sequence determinants for the enanatioselectivity of the murine 12R-LOX. Alignments of known S-and R-LOXs led to the identification of a single amino acid at position 441 in the mouse 12R-LOX, which is always an alanine in the S-LOX but glycine in R-LOXs. When Gly441 was mutated to alanine and valine in murine 12R-LOX we obtained the following results: the G441A mutant oxygenated arachidonic acid methyl ester more to 8S-HETE. With the wild-type enzyme 8-HETE was only a minor side product and its chirality was exclusively R. When linoleic acid methyl ester was used as substrate, there was a drastic change in the product pattern from 9R-HODE towards 13S-HODE. Unfortunately, the G441V mutant was enzymatically inactive with either of the substrates.

The above results suggest that with the G441A mutant there might be a stearic hindrance of oxygen insertion at C12 (arachidonic acid methyl ester) and C9 (linoleic acid methyl ester). Glycine is a very small amino acid and may not hinder oxygen insertion. In contrast, when glycine is mutated to alanine, there is a more bulky side chain, which might mask the site of oxygen insertion at C-12 and forcing insertion of molecular oxygen at C-8 thereby forming more of 8S-HETE than in the wild type enzyme. It is remarkable, that relatively subtle alterations in the side-chain geometry of G441 led to pronounced effects in the stereochemistry of the LOX reaction. Addition of a single CH_2-group (G441A) led to dramatic alterations in the enzyme specificity and addition of a further CH_2-group (G441V) to an inactive enzyme species.

4.5. DETERMINANTS OF SUBSTRATE SPECIFICITY:

The mouse 12R-lipoxygenase is expressed preferentially in the skin and was found to be involved in the embryonic skin development. The murine

12R-LOX exhibits peculiar characteristics, which distincts this isoenzyme from most other LOX-isoforms. One of these peculiarties is its substrate preference towards arachidonic acid methyl ester and other methylated substrates while the other LOXs prefer free fatty acids. One way to explain this mechanistic difference is to assume an inverse substrate alignment at the active site. In this case the substrate fatty acid may slide into the substrate binding pocket with its carboxylate ahead. Burying a strongly polar free carboxylate inside the hydrophobic environment of the substrate-binding pocket may be thermodynamically hindered and thus, free fatty acids are not accepted as substrates for this LOX isoform. However, carboxylate methylation reduces the polarity of this residue and thus, may decrease thermodynamic hindrance.

As indicated above the murine 12R-LOX strongly prefers arachidonic acid methyl ester to the free acid, whereas the human enzyme accepts both substrates. To explore the structural reasons for this different substrate specificity we aligned the two sequences and observed a high degree of sequence conservation (86% conserved residues). However, in the C-terminal part of the enzyme we found an accumulation of sequence differences. These data prompted us to construct a chimeric enzyme from the murine 12R-LOX, which contained a 500 bp fragment of the C-terminal region of the human 12R-LOX. When this chimer was reacted with free arachidonic acid, it showed strong activity towards this substrate. Thus, it may be concluded that the C-terminal part of the human 12R-LOX contain amino acid residues, which are responsible for accepting free arachidonic acid as substrate. When we inspected the sequence of the 500 bp fragment an arginine was identified, which is a hydrophobic residue in the murine enzyme. If this arginine is a constituent of the active site it may contribute to stabilize the enzyme substrate complex by interacting with the substrate's free carboxylate. Site directed mutagenesis of this residue is on the way to provide experimental evidence for this hypothesis.

4.5. IRON LIGANDS:

X-ray analyses of crystals of soybean lipoxygenase L-1 (Boyington et al., 1993; Minor et al., 1996), soybean lipoxygenase L-3 (Skrzypczak-Jankun et al., 1997), and the rabbit of 15-lipoxygenase (Gillmor et al., 1997)) identified three histidines and the carboxylate of the C-terminal isoleucine as proteinogenic iron ligands. While all the crystal structures show four ligands in common, some of the structures in the soybean lipoxygenase L-1 and the rabbit 15-LOX show a fifth proteinogenic iron ligand. (Minor et al., 1996; Gillmor et al., 1997). This putative fifth iron ligand of lipoxygenases is conserved as Asn, His or Serine (Matsumoto et al., 1988; Sigal et al., 1988; Jisaka et al., 2001). In mammalian 12R-LOX the fifth ligand is an asparagine. We have mutated this amino acid in murine 12R-LOX to histidine and serine. These experiments did not result in a dramatic change in the specific activities. The specific activity of the N582S was about half of the wild type and the specific activity of the N582H remained same as that of the wild type. An interesting alteration in positional specificity was observed for the N582H mutant. This amino acid exchange led to an enzyme species, which converted arachidonic acid methyl ester mainly to 11R-HETE. The mechanistic reason for this unexpected result remains unclear. To understand this phenomenon it is essential to find out whether or not N528 does act as immediate iron ligand.

4.6. EXTRA DOMAIN IN R-LOXs:

The structure of the 12R-LOX genes is unique among all animal lipoxygenases since a proline-rich 31 amino acid insert is present in exon 4. Structural modelling suggested that this extra domain forms an unstructured loop between helix 1 and 2 at the surface of the C-terminal catalytic domain close to the entrance into the substrate binding pocket (Krieg et al., 1999). Removal of this amino acid stretch in human 12R-LOX resulted in the complete loss in activity (Schneider and Brash, 2002). We have removed this stretch in the mouse 12R-LOX and also performed deletion mutations of the

first and the last five amino acid residues of this stretch. Complete removal of this stretch (P154-P185) resulted in about 90 % loss of enzyme activity. Similarly removal of first 5 amino acids P154-I159 resulted in the complete loss of activity while the deletion of the last 5 amino acids (Y180-P185) did neither dramatically alter the activity nor the specificity of the enzyme. This indicates that the first 5 amino acids of this domain maybe important in the conformation of the tertiary structure of the enzyme. The deletion of the first 5 amino acids may also result in the wrong conformational folding of the protein with loss of activity.

CONCLUSIONS

Lipoxygenases (LOXs) form a heterogeneous family of lipid peroxidizing enzymes, which catalyze dioxygenation of free and/or esterified polyunsaturated fatty acids to their corresponding hydroperoxy derivatives. These products are involved in the biosynthesis of inflammatory mediators, such as leukotrienes and lipoxins, and have been implicated in cell differentiation, carcinoma metastasis, atherogenesis and osteoporosis. In mammals, LOXs are categorized with respect to their positional specificity of arachidonic acid oxygenation into 5-, 8-, 12- and 15-LOXs.

Most of the mechanistic studies on the positional specificity of mammalian LOXs have been carried out on the classical S-LOX, such as the rabbit and human 15-LOX-2, the leukocyte-type 12-LOX and the human 5-LOX. Unfortunately, little is known at the moment about the stereochemical control mechanisms of the recently discovered R-LOXs.

To investigate the structural basis for the stereochemical control mechanisms in mammalian R-LOXs in more detail we applied an integrated research strategy involving targeted substrate modification and site-directed mutagenesis in murine 12R-LOX.

The murine 12R-LOX was first expressed in *E.Coli*, exhibiting a specific activity of 10 µg HETE formation/mg LOX protein during a 15 min incubation period. This value was about three orders of magnitude lower than that of the specific activity determined for the rabbit 15-LOX using free arachidonic acid as substrate. One possible explanation for this discrepancy was that the enzyme might require post-translational modification for its activity. To test this hypothesis we expressed this protein in the eukaryotic expression system and observed an increase (4-fold) in the specific activity (38 µg HETE/mg protein during a 15 min incubation period). Thus, post-translational modifications are important for the specific activity of this enzyme species.

The structural basis for the reaction specificity of various S-LOX isoforms has been investigated in detail and various sequence determinants for the positional specificity have been identified. To investigate the amino acid residues involved in the control of 12R-LOX reaction we first identified the amino acids Phe390,

Ala455 and Val631 in murine 12R-LOX which corresponds with the sequence determinants for the positional specificity of other mammalian LOX-isoforms.

When Phe390 and Ala455 were mutated to more space-filling or less bulky amino acids there was unspecific product pattern indicating that the mutant enzyme species have lost their stereochemical control mechanisms. Similar alterations were observed when Val631 was mutated to more space filling Ile and Phe.

When Val631 was mutated to less bulky residues (Ala or Gly) there was an increase in the specific activity and specific alterations in the product pattern. A major 11-HETE formation which was enantioselective suggested a tight stereochemical control of the reaction. Formation of 12R-HETE involves C-10 hydrogen abstraction and [+2]-radical rearrangement. In contrast, synthesis of 11R-HETE requires C-13 hydrogen abstraction and [-2]-radical rearrangement. This data is explained if the substrate fatty acid slides into the substrate binding pocket with its carboxylate ahead.

But when the modified substrate 20-HETE methyl ester was used, we obtained 8, 20-diHETE and 9, 20-diHETE methyl esters for the wild type and V631A mutant respectively. 8, 20-diHETE formation involves C-10 hydrogen removal and [-2]-radical rearrangement. In contrast, 9, 20-diHETE formation involves C7 hydrogen removal and [+2]-radical rearrangement. This data suggests that the 20-HETE methyl ester may slide into the substrate binding cleft with its methyl end first.

The structural reasons for these mechanistic differences appear to be complex and cannot be explained solely on the basis of the space or orientation related hypothesis of the positional specificity. It may be concluded that substrate fatty acids can adopt different catalytically productive arrangements within the active site of the murine 12R-LOX and each of these arrangements may lead to the formation of different oxygenation products.

Multiple sequence alignment of R- and S-lipoxygenases indicated a conserved Ala in the mammalian S-LOXs. In R-LOXs this Ala was substituted by a Gly. When Gly441 of the murine 12R-LOX was mutated to Ala, the mutant enzyme produced more 8S-HETE as opposed to 8R-HETE, which was the minor product of the wild type LOX. With linoleic acid methyl ester there is a drastic

shift in the product pattern since the wild type produces mainly 9R-HODE while the 13S-HODE was analyzed as the main product of the G441A mutant. As mechanistic interpretation of these data it is suggested that Ala substituted at this position might shield C-12 so that oxygen insertion might shift towards the C-8 leading to an increase in the 8S-HETE formation. The same applies to the formation of products with linoleic acid methyl ester.

The preference of murine 12R-LOX towards the arachidonic acid methyl ester was found to be due to the substrate determinants present towards the C-terminal region of the enzyme. Substitution of this region with the human 12R-LOX lead to a chimeric enzyme which showed activity towards free arachidonic acid. One of the substrate determinant identified is an arginine which may contribute to stabilize the enzyme substrate complex by interacting with the substrate`s free carboxylate.

The putative fifth iron ligand of lipoxygenases is conserved as Asn, His or Ser. In mammalian 12R-LOX the fifth iron ligand is an asparagine and the substitution of this residue to serine and histidine lead to catalytically active enzymes. There is no change in the product pattern of N582S mutant while the N582H showed an increase in the 11R-HETE formation. This infers that the substitution of serine at this position was tolerated while the histidine substitution lead to a minor disturbance in enzyme structure forming altered product pattern.

The R-lipoxygenases are unique in having an extra stretch of proline rich amino acids which do not have sequence homology to any protein. Removal of this entire stretch and also the first five amino acids of the stretch led to an inactive enzyme species indicating the importance of the first five amino acids in the proper folding of the 12R-LOX.

The conclusions of the present study are:

- 12(R)-LOXs form a separate LOX-subfamily, which contain a different set of sequence determinants for the positional specificity than the S-LOXs.

- Fatty acid substrates are inversely aligned (head to tail orientation) at the active site of 12R-LOXs i.e., the carboxylate end of the substrate penetrates into the active site.

- V631 is a sequence determinant for regiospecificity of the murine 12(R)-LOX. Introduction of less space filling residues at this position converts the 12R-LOX into an 11R-lipoxygenating enzyme

- G441 was found to be a sequence determinant for stereospecificity of R- and S-LOXs.

- Mutation of N582, the putative fifth iron ligand to an iron liganding H, altered the positional specificity of the enzyme in favour of 11-oxygenation.

- The proline-rich stretch of 31 amino acids in the mouse 12R-LOX is needed for proper conformation of the enzyme and for the enzyme activity.

- Differences in substrate preference of human and mouse 12(R)-LOXs may depend on the amino acid sequence of the C-terminal part of the primary structure.

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