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REVIEW ARTICLE

Biosynthesis and metabolism of leukotrienes

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Leukotrienes are metabolites of arachidonic acid derived from the action of 5-LO (5-lipoxygenase). The immediate product of 5-LO is LTA_4 (leukotriene A_4), which is enzymatically converted into either LTB_4 (leukotriene B_4) by LTA_4 hydrolase or LTC_4 (leukotriene C_4) by LTC_4 synthase. The regulation of leukotriene production occurs at various levels, including expression of 5-LO, translocation of 5-LO to the perinuclear region and phosphorylation to either enhance or inhibit the activity of 5-LO. Several other proteins, including $cPLA_2\alpha$ (cytosolic phospholipase $A_2\alpha$) and FLAP (5-LO-activating protein) also assemble at the perinuclear region before production of LTA_4. LTC_4 synthase is an integral membrane protein that is present at the nuclear envelope; however, LTA_4 hydrolase remains cytosolic. Biologically active LTB_4 is metabolized by ω-oxidation carried out by specific cytochrome P450s (CYP4F) followed by β-oxidation from the ω-carboxy position and after CoA ester formation. Other specific pathways of leukotriene metabolism include the 12-hydroxydehydrogenase/15-oxo-prostaglandin-13-reductase that forms a series of conjugated diene metabolites that have been observed to be excreted into human urine. Metabolism of LTC_4 occurs by sequential peptide cleavage reactions involving a γ-glutamyl transpeptidase that forms LTD_4 (leukotriene D_4) and a membrane-bound dipeptidase that converts LTD_4 into LTE_4 (leukotriene E_4) before ω-oxidation. These metabolic transformations of the primary leukotrienes are critical for termination of their biological activity, and defects in expression of participating enzymes may be involved in specific genetic disease.

Key words: γ-glutamyl leukotrienase, leukotriene A_4 hydrolase (LTA_4 hydrolase), leukotriene C_4 synthase (LTC_4 synthase), 5-lipoxygenase (5-LO), 5-lipoxygenase-activating protein (FLAP), membrane-bound dipeptidase.

INTRODUCTION

The co-ordination of biochemical events between cells in a tissue or in an intact organism plays a central role in the response of each cell to external or internal stimuli. A large number of molecular substances have been selected to serve the role of cellular mediators. Various mechanisms have also evolved that recognize small molecules as signalling species. The metabolites of arachidonic acid constitute a family of cellular communicators, often termed eicosanoids. These eicosanoids are recognized by GPCRs (G-protein-coupled receptors), nuclear receptors and by proteins that facilitate transfer of biochemical intermediates from one cell to another. Leukotrienes are a unique family of eicosanoids derived from arachidonic acid whose synthesis and metabolism will be the focus of this review.

One level of understanding the complex structural alteration of arachidonic acid into the covalent arrangement of atoms called leukotrienes culminated from structural studies carried out in the laboratory of Dr Bengt Samuelsson in the late 1970s with the characterization of novel arachidonate metabolites generated by human PMNNs (polymorphonuclear neutrophils) [1] and the structural characterization of the biologically active compound, termed slow-reacting substance of anaphylaxis (SRS-A) [2]. These studies led to the recognition of a novel pathway of oxidation of arachidonic acid catalysed by an enzyme called 5-lipoxygenase (5-LO). This enzyme is responsible for the formation of several conjugated triene metabolites containing a linear 20-carbon chain which have profound biological activities including that of contracting smooth muscle (SRS-A) [3] or causing neutrophil chemotaxis [4]. The term leukotriene was coined to indicate the presence of three conjugated double bonds within the 20-carbon structure of arachidonic acid as well as the fact that these compounds were derived from leucocytes such as PMNNs or transformed mast cells. Interestingly, most of the cells known to express 5-LO are of myeloid origin, which includes neutrophils, eosinophils, mast cells, macrophages, basophils and monocytes. Remarkable progress has been made in understanding the individual biochemical steps that take place to generate the biologically active leukotrienes, and to metabolically inactivate these products. Yet as progress has been made in unravelling each event, new layers of complexity have emerged. These complexities include the phospholipases engaged in releasing arachidonic acid, regulation of 5-LO, suicide inactivation, translocation of critical proteins to the perinuclear region, phosphorylation and Ca^{2+}-dependent activation, as well as recognition of associated proteins that appear to assemble to form a biochemical machine that generates leukotrienes. The subcellular location of critical enzymes involved in leukotriene biosynthesis is summarized in Table 1. Perhaps

Abbreviations used: ADH, alcohol dehydrogenase; AldDH, aldehyde dehydrogenase; BLT, leukotriene B_4 receptor; $cPLA_2$, cytosolic phospholipase A_2; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CLP, coactosin-like protein; CysLT, cysteinyl leukotriene; FLAP, 5-lipoxygenase-activating protein; GGL, γ-glutamyl leukotrienase; GGT, γ-glutamyl transpeptidase; GGT-rel, GGT-related; GPCho, glycerophosphocholine; GPCR, G-protein-coupled receptor; 12HDH/15oPGR, 12-hydroxydehydrogenase/15-oxo-prostaglandin-13-reductase; 10-HOTrE, 10-hydroxyoctadecatrienoic acid; 5-HpETE, 5-hydroperoxy-eicosatetraenoic acid; $iPLA_2$, intracellular calcium-independent phospholipase A_2; LAT, lysophospholipid:acyl-CoA acyltransferase; LO, lipoxygenase; LOOH, lipid hydroperoxide; LTA_4, leukotriene A_4; LTA_4-H, leukotriene A_4 hydrolase; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; LTC_4-S, leukotriene C_4 synthase; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4; MAPEG, membrane-associated proteins in eicosanoid and glutathione metabolism; MBD, membrane-bound dipeptidase; mGST, microsomal glutathione S-transferase; PAF, platelet-activating factor; PGE, prostaglandin E; PKC, protein kinase C; PLA_2, phospholipase A_2; PMNN, polymorphonuclear neutrophil; $sPLA_2$, secretory PLA_2; SRS-A, slow-reacting substance of anaphylaxis; TGFβ, transforming growth factor β.

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Table 1 Subcellular location of enzymes involved in synthesis and metabolism of LTA_4 from cellular phospholipid precursors

Protein	Resting cell location	Activated cell location
$cPLA_2\alpha$	Cytosol	Perinuclear membrane, Golgi apparatus, endoplasmic reticulum
5-LO	Cytosol, nucleoplasm	Perinuclear membrane
FLAP	Perinuclear membrane	Perinuclear membrane
LTA_4-H	Cytosol	Cytosol, nucleoplasm
LTC_4-S	Perinuclear membrane	Perinuclear membrane

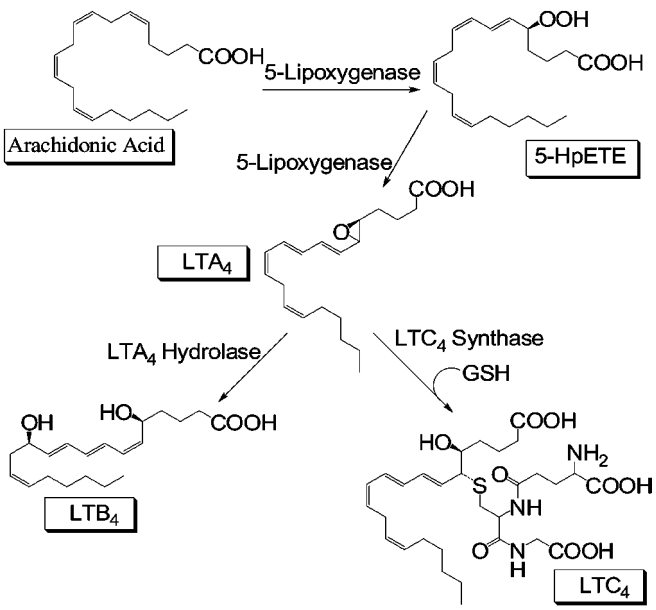

Figure 1 Biochemical pathway for the conversion of arachidonic acid into biologically active LTB_4 and LTC_4 catalysed by 5-LO

The structures of the intermediates 5-HpETE and LTA_4, as well as the biologically active LTB_4 and LTC_4 are indicated.

even more surprising was that a chemically reactive intermediate, LTA_4 [leukotriene A_4, 5(*S*)5,6-oxido-7,9,11,14-(*E*,*E*,*Z*,*Z*)-eicosatetraenoic acid], is generated in the biosynthetic process and can be transported from one cell to another and continue its biochemical transformation in spite of its very short chemical half-life.

Leukotriene biosynthesis begins with the specific oxidation of arachidonic acid by a free radical mechanism as a consequence of interaction with 5-LO (Figure 1). The first enzymatic step involves the abstraction of a hydrogen atom from C-7 of arachidonate followed by the addition of molecular oxygen to form 5-HpETE (5-hydroperoxyeicosatetraenoic acid). A second enzymatic step is also catalysed by 5-LO and involves removal of a hydrogen atom from C-10, resulting in formation of the conjugated triene epoxide LTA_4. LTA_4 must then be released by 5-LO and encounter either LTA_4-H (LTA_4 hydrolase) or LTC_4-S [LTC_4 (leukotriene C_4) synthase]. LTA_4-H can stereospecifically add water to C-12 while retaining a specific double-bond geometry, leading to LTB_4 [leukotriene B_4, 5(*S*),12(*R*)-dihydroxy-6,8,10,14-(*Z*,*E*,*E*,*Z*)-eicosatetraenoic acid]. If LTA_4 encounters LTC_4-S, then the reactive epoxide is opened at C-6 by the thiol anion of glutathione to form the product LTC_4 [5(*S*)-hydroxy-6(*R*)-*S*-glutathyionyl-7,9,11,14-(*E*,*E*,*Z*,*Z*)-eicosatetraenoic acid], essentially a glutathionyl adduct of oxidized arachidonic acid. Both of these terminal leukotrienes are biologically active in that specific GPCRs recognize these chemical structures and receptor recognition initiates complex intracellular signalling cascades. In order for these molecules to serve as lipid mediators, however, they must be released from the biosynthetic cell into the extracellular milieu so that they can encounter the corresponding GPCRs. Termination of the biological activity of these primary leukotrienes is a consequence of metabolic conversion into a series of products which are not recognized by GPCRs. A host of pathways are now known to be engaged to carry out these metabolic transformations. Even though the chemical transformation of arachidonic acid into LTB_4 and LTC_4 was described 25 years ago, the complexity involved in regulation of this complete process has continued to emerge.

The leukotriene GPCRs have now been cloned and expressed, and have been designated BLT-1 and BLT-2 that bind LTB_4 specifically, and $CysLT_1$ and $CysLT_2$ which are known to bind the CysLTs (cysteinyl leukotrienes) LTC_4, LTD_4 [leukotriene D_4, 5(*S*)-hydroxy-6(*R*)-*S*-cysteinylglycyl-7,9,11,14-(*E*,*E*,*Z*,*Z*)-eicosatetraenoic acid] and LTE_4 [leukotriene E_4, 5(*S*)-hydroxy-6(*R*)-*S*-cysteinyl-7,9,11,14-(*E*,*E*,*Z*,*Z*)-eicosatetraenoic acid]. Recently, a novel receptor that binds both UDP and LTC_4 has been described as GPCR17 [5]. There have been excellent reviews in recent years describing the characterization of these leukotriene GPCRs, as well as pharmacological antagonism [6–11].

This review of leukotriene biosynthesis will outline our understanding of the engagement of phospholipases to release free arachidonic acid and then detail our understanding of each enzymatic step and cellular events that are involved in leukotriene production. This will be followed by a description of the known metabolic pathways that terminate the action of leukotrienes.

PLA_2s (PHOSPHOLIPASES A_2) AND ACYLTRANSFERASES

Membrane glycerophospholipids in eukaryotic cells frequently contain an unsaturated fatty acyl moiety esterified at the *sn*−2 position. Polyunsaturated fatty acids such as arachidonic acid are especially abundant in certain types of cells, including those of myeloid origin such as PMNNs or macrophages. The initial step in the synthesis of leukotrienes is the cleavage of this arachidonoyl ester bond through the action of a PLA_2 in a hydrolysis reaction that yields lysophospholipid and free arachidonic acid. Cellular levels of both arachidonic acid and lysophospholipids are tightly regulated. One of the main mechanisms that assists in maintaining low levels of these bioactive molecules is the phospholipid reacylation process often termed the Lands cycle [12]. In this pathway (Figure 2), fatty acids are eventually conjugated with CoA through the action of fatty acyl-CoA ligases, and fatty acyl-CoA esters are then esterified to lysophospholipids in a reaction catalysed by LAT (lysophospholipid:acyl-CoA acyltransferase). Data from this [13] and other groups [14] support the essential role of this reacylation process in regulating the availability of free arachidonic acid and the subsequent production of eicosanoids after cell stimulation. For instance, inhibition of LAT with the organomercury compound thimerosal results in a dramatic increase in LTB_4 released by PMNNs stimulated with GM-CSF (granulocyte/macrophage colony-stimulating factor) and fMLP (*N*-formylmethionyl-leucyl-phenylalanine) [13]. Although acyltransferase enzyme activities have been measured and studied for many years, the proteins responsible for the reacylation of arachidonic acid remain to be identified. Recently, two groups independently cloned a LAT that is preferentially expressed in rat and mouse lung, although this enzyme exhibits preference for saturated fatty acids, and is therefore unlikely to be involved in the reacylation of arachidonic acid [15,16]. An additional enzyme, termed LPCAT2,

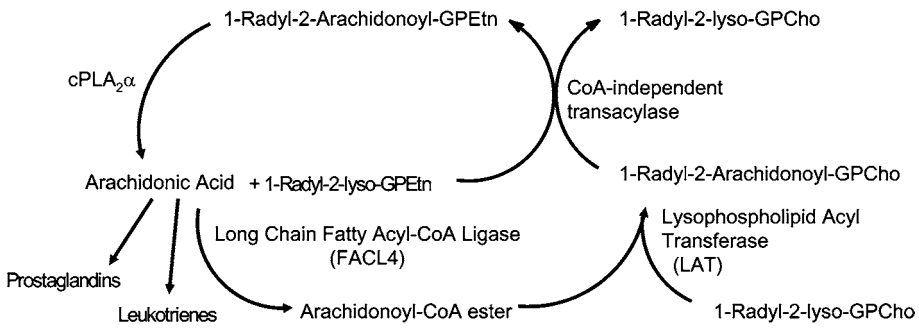

Figure 2 Remodelling of arachidonic acid (Lands cycle) is initiated by activation of cPLA$_2\alpha$ which liberates a lysophospholipid

The lysophospholipid liberated is probably lysoglycerophosphoethanolamine lipid according to studies of the liberation of arachidonate within the human neutrophil [163]. A portion of the liberated arachidonic acid is converted into arachidonoyl-CoA ester by long-chain fatty acyl-CoA ligase. The CoA ester and a lysoglycerophosphocholine lipid species can react by the action of lysophospholipid acyltransferase to re-esterify arachidonic acid. Movement of the arachidonic acid into phosphatidylethanolamine phospholipids is possibly carried by a CoA-independent transacylase enzyme [164].

which is an acetyl CoA:lyso-PAF (platelet-activating factor) acetyltransferase, has also been cloned and shown to be able to incorporate arachidonoyl-CoA into phosphatidylcholine [17]. The relevance of this novel enzyme in eicosanoid biosynthesis remains to be determined.

Different PLA$_2$ enzymes have been identified and classified into 15 different groups according to their primary structures, localization and properties such as Ca^{2+} ion requirements [18]. Several PLA$_2$ groups include distinct isoforms. It is useful to distinguish five separate families of mammalian PLA$_2$s: low-molecular-mass calcium-dependent secretory PLA$_2$s (sPLA$_2$s), which includes groups I, II, III, V, X and XII; calcium-dependent cytosolic PLA$_2$ and its paralogues (cPLA$_2$s), which includes group IV; intracellular calcium-independent PLA$_2$s (iPLA$_2$s), which include group VI; calcium-independent PAF-acetylhydrolase, which includes groups VII (also referred to in recent literature as lipoprotein-associated PLA$_2$ or Lp-PLA$_2$) and VIII; and the recently identified lysosomal PLA$_2$ (group XV).

Although different PLA$_2$s, especially various sPLA$_2$s (in some cases through binding to extracellular receptors) and iPLA$_2$s have been shown to initiate or amplify arachidonic acid release in different experimental models, it is widely recognized that cPLA$_2\alpha$ (or Group IVA PLA$_2$) plays a major role in the initial activation-dependent events that lead ultimately to leukotriene production. The extensive experimental data that support this major role of cPLA$_2\alpha$ in eicosanoid production have been reviewed elsewhere [19], but the central role that cPLA$_2\alpha$ plays in the production of leukotrienes was demonstrated by the fact that cells from transgenic mice deficient in this enzyme showed an almost complete inability to synthesize prostaglandins or leukotrienes in response to a variety of stimuli [20–22].

Among all the PLA$_2$ enzymes, cPLA$_2\alpha$ is unique in showing a marked substrate preference for glycerophospholipids containing arachidonic acid esterified at the *sn*−2 position [23,24]. Additionally, it also possesses lysophospholipase activity. The crystal structure of cPLA$_2\alpha$ revealed an N-terminal C2 domain, homologous in structure and function with the C2 domain present in PKC (protein kinase C), and a C-terminal catalytic domain [25]. Ca^{2+} ions play a critical role in the activation of cPLA$_2\alpha$, a cytosolic enzyme in resting cells, in that it rapidly translocates to intracellular membranes (Golgi apparatus, endoplasmic reticulum and nuclear envelope) upon elevation of the cytosolic Ca^{2+} concentration. Recombinant cPLA$_2\alpha$ lacking the N-terminal C2 domain responsible for the binding of Ca^{2+}, or with mutations of critical Ca^{2+}-binding amino acids such as Asp^{43} or Asp^{93}, were unable to translocate to membranes or to release arachidonic acid [26]. The catalytic domain contains the dyad formed by Ser^{228} and Asp^{549}, located at the end of a deep funnel, where a single molecule of phospholipid can diffuse after enzyme binding to membranes or to phospholipid vesicles. Arg^{200} helps to stabilize the phospholipid substrate through ionic interaction with the phosphate group. The catalytic mechanism involves an acyl-serine intermediate formed after the nucleophilic attack of the *sn*–2 ester bond by Ser^{228}. The catalytic domain also contains three serine residues which have been shown to be phosphorylated upon cell activation and involved in increased arachidonic acid release [24].

The regulation of cPLA$_2\alpha$ is very complex and is still not completely understood. cPLA$_2\alpha$ is present at different levels in many tissues, but its expression can be modulated in response to stimuli such as cytokines, growth factors or corticosteroids. Its activity is also the target of a variety of regulatory mechanisms that include phosphorylation by different kinases, interaction with lipids such as ceramide 1-phosphate or phosphoinositides, interaction with proteins such as annexin or vimentin, or cleavage catalysed by caspases [23,24]. One aspect of particular interest is the intracellular localization of cPLA$_2\alpha$ and its functional interaction with downstream proteins involved in leukotriene biosynthesis, as will be discussed below.

5-LIPOXYGENASE

The enzymatic addition of molecular oxygen to polyunsaturated fatty acids is a reaction that occurs in the plant as well as the animal kingdoms. The three-dimensional structure of several LOs, including soya bean 15-LO (15-lipoxygenase) [27,28], a coral 8-LO (8-lipoxygenase) [29] and rabbit reticulocyte 15-LO [30] have been determined and have provided considerable insight into this class of enzymes. These LOs all have very similar three-dimensional structures in spite of only modest sequence homology. The structure of 5-LO has not been reported, even though it was first purified and cloned almost 20 years ago [31]. Human 5-LO has only 57 % identity with rabbit 15-LO, but on the basis of structural similarities between the known plant and animal 15-LOs, attempts have been made to model 5-LO upon the rabbit 15-LO three-dimensional structure (Figure 3) [32].

Some rather important features have emerged from model studies in that a two-domain structure appears to be present with a small N-terminal C2-like domain critical to membrane binding of 5-LO during activation. This domain has many similarities to lipases, including cPLA$_2\alpha$ (mentioned above) and binds two Ca^{2+} ions. This C2-like domain of 5-LO is highly negatively charged, and binding of Ca^{2+} is thought to neutralize this charge [33].

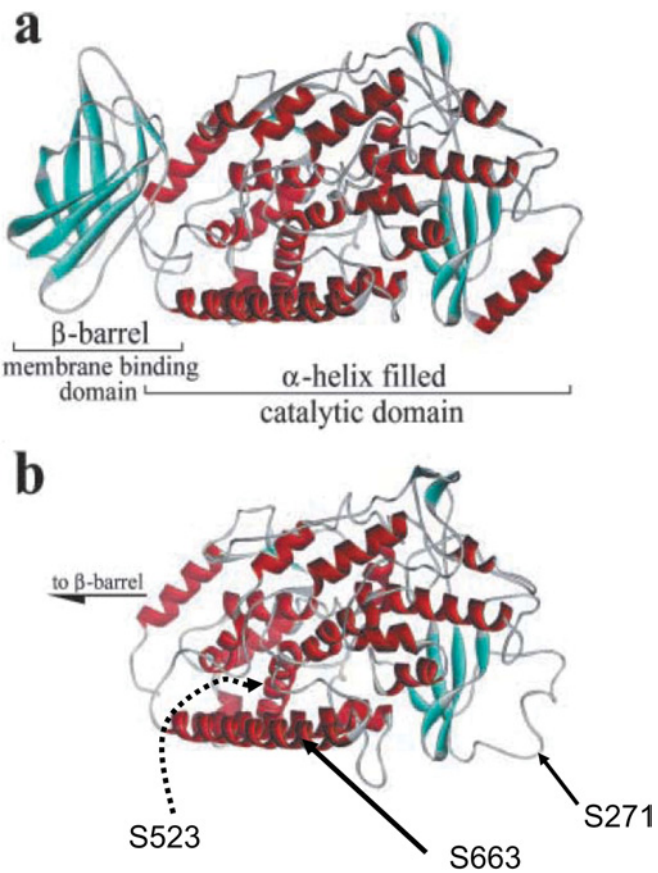

Figure 3 Comparison of the theoretical model for the catalytic domain of 5-LO with the resolved structure of 15-LO

(**A**) Entire 15-LO molecule from PDB code 1LOX. (**B**) Theoretical model of the catalytic domain of human 5-LO. Sites of phosphorylation on the catalytic domain are indicated. Reproduced from Journal of Molecular Modeling, vol. 8, 2002, pp. 102–112, 'Structural characterization of the catalytic domain of the human 5-LO enzyme' by J. Hemak, D. Gale and T. G. Brock, Figure 2(b) © Springer with kind permission of Springer Science and Business Media.

The Ca^{2+} binding at the C2-like domain also appears to change orientation of three tryptophan side chains (Trp^{13}, Trp^{75} and Trp^{102}) so as to maximize membrane insertion potential. After Ca^{2+} binding, this enzyme increases its affinity for certain membrane phospholipids, specifically binding to fluid GPCho (glycerophosphocholine) molecular species typically found within the nuclear membrane [34]. Such phospholipid classes are not particularly abundant on the inner leaflet of the cellular plasma membrane, which has more anionic bulk character because of higher abundance of glycerophosphoserine and glycerophosphoinositol present at the inner leaflet [35]. Other proteins which have C2 domains (PKCβ and phospholipase Cδ1) are known to translocate and bind rather specifically to the plasma membrane inner leaflet because of affinity for these acidic phospholipids [36]. This unique affinity of 5-LO and cPLA$_2\alpha$ for GPCho lipids has been suggested to account for binding to the perinuclear region of the cell once activated by elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

The C-terminus of 5-LO contains the catalytic site with an iron ion chelated by three histidine residues (His^{367}, His^{372} and His^{550}), as well as Asn^{554} and the isoleucine carboxy group at the C-terminus (Ile^{673}), based on modelling studies [37]. There is substantial conserved structure in this domain of LOs across both animal and plant kingdoms which has been used to propose the 5-LO tertiary structure as well as its catalytic mechanism. The arachidonate-binding site for LOs is thought to be a 'U-shaped' region which places the essential Fe^{3+} ion in close proximity to the critical bisallylic hydrogen atom that is engaged in the initial hydrogen abstraction process [38]. Structural similarity of all LOs has been supported by the ability to convert 15-LO into 12-LO (12-lipoxygenase) [38,39] and 5-LO into 8-LO and 15-LO activities [40], as well as 15-LO being a LTA_4 synthase [41].

Recently, details of the catalytic cycle of human and soya bean LO have been proposed based on X-ray crystallography, site-directed mutagenesis, EPR studies and spectroscopic measurements [42,43]. In line with structural similarities of the different LOs, a catalytic cycle for 5-LO can be proposed as in Figure 4. This hypothetical cycle would begin with cytosolic 5-LO that contains iron in the ferrous state, and with water as the sixth ligand of the co-ordination sphere. This form of the enzyme would be active as a peroxidase because Fe^{2+} could reduce a lipid hydroperoxide (LOOH), generating active Fe^{3+} in the active site, now with a hydroxide ion as the sixth co-ordination ligand. By a hydrogen tunnelling mechanism which requires proximity of approx. 0.6–0.8 Å (1 Å = 0.1 nm) between Fe^{3+} and the proS hydrogen C-7 bond atom of arachidonate [44], an electron can enter into the d-orbitals of Fe^{3+} leaving the OH^- ion ligand to trap the nascently forming proton (H^+) and leaving a radical site at C-7 of arachidonic acid. Molecular oxygen could then attack C-5 of the pentadienyl radical forming the 5-hydroperoxy radical intermediate in the active site. The resulting Fe^{2+} ion can now donate an electron to the oxygen-centred radical to form the 5-hydroperoxy anion. This anion would attack the proton from water in the sixth ligand position, regenerating the active Fe^{3+} ion with OH^- in its co-ordination sphere and releasing 5-HpETE. An interesting feature of this mechanism in contrast with cyclo-oxygenase, which also carries out free radical oxidation of arachidonic acid, is that regeneration of the active form of 5-LO would occur during the catalytic cycle of 5-HpETE formation without the need to have a separate lipid hydroperoxide reduction step that is required in the redox cycle of cyclo-oxygenase and its haem-bound iron [45].

Interference with the ligand sphere of iron in the active site of 5-LO has been a drug target. Hydroxyurea-based drugs have been developed that can specifically inhibit 5-LO, most likely by chelating active-site iron [46]. Another feature of 5-LO is that the suspected arachidonate-binding channel is approx. 20 % larger than it is in 15-LO, which probably alters the orientation of arachidonic acid during initial binding [47] and perhaps 5-HpETE binding within the active site so that the second mechanism of 5-LO can take place. In this second reaction of LTA_4 biosynthesis by 5-LO, the catalytic cycle is repeated. The initial removal of H^+ and abstraction of an electron are initiated now at C-10 of 5-HpETE with formation of an extended hexatrienyl radical site. This results in emergence of high electron density at C-6 of 5-HpETE which profoundly affects the first oxygen atom of the 5-hydroperoxide moiety. The oxygen–oxygen bond is broken by the donation of an electron from Fe^{2+} and the triene epoxide product is released as a OH^- ion is formed. The OH^- ion can then abstract the proton from the water in the sixth ligand of Fe^{3+}, completing the reaction cycle by reforming the Fe^{3+} iron with OH^- ion bound in its co-ordination sphere.

Regulation of 5-LO activity

Alteration of the ability of cells that express 5-LO to synthesize leukotrienes occurs by numerous mechanisms. As described above, elevation of $[Ca^{2+}]_i$ has a fundamental effect on the hydrophobicity of cPLA$_2\alpha$ and 5-LO and their translocation to the perinuclear region that contains glycerophospholipids with esterified

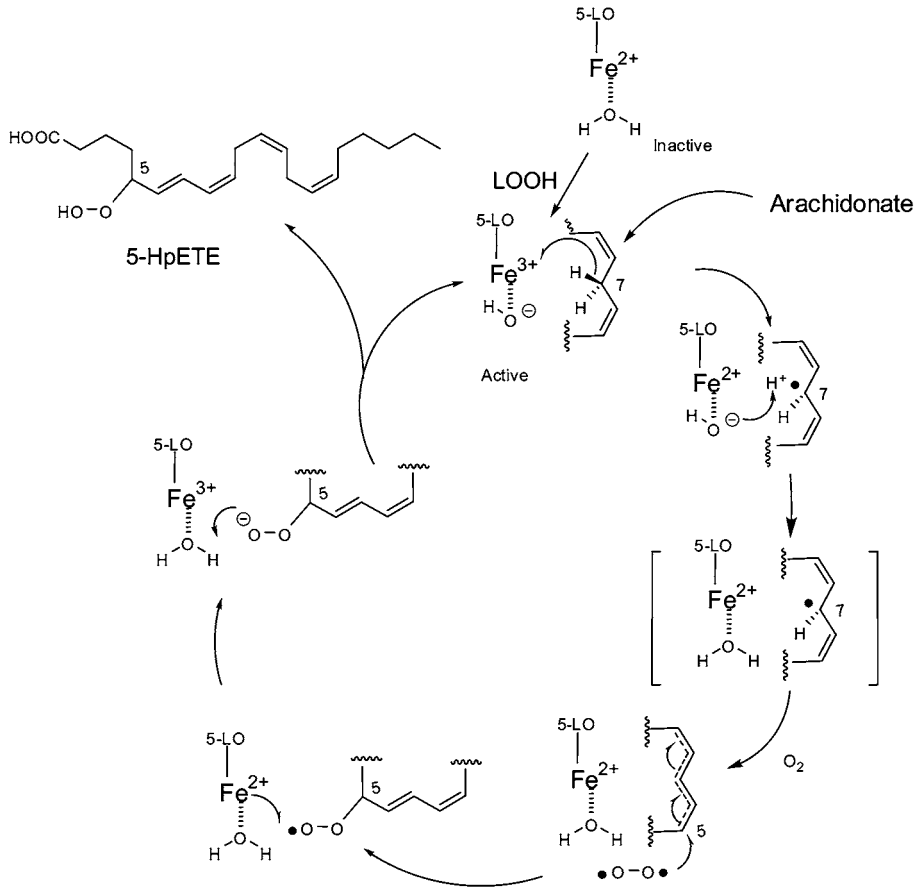

Figure 4 Catalytic cycle of 5-LO

The cycle starts from the inactive Fe^{2+} ion in the active site with water as the sixth ligand, 5-LO is activated by reduction of an LOOH to yield the active 5-LO containing Fe^{3+} iron in the active site and a hydroxide anion as the sixth ligand. The catalytic cycle is completed as described in the text to convert arachidonic acid into 5-HpETE.

arachidonic acid. In large part, this translocation event has been thought to be controlled by a charge neutralization mechanism of the C2-like domain, exposing critical tryptophan residues that become embedded in the membrane bilayer. However, recent evidence has suggested that the substrate, arachidonic acid, may play an important role in altering the fundamental hydrophobic properties of cytosolic 5-LO separately from these Ca^{2+} effects. It is possible that binding of arachidonic acid to cytosolic 5-LO can substantially alter the affinity of 5-LO for the nuclear bilayer [48].

Phosphorylation of 5-LO also plays a role in regulating enzymatic activity. Three separate sites have been identified: a MAPKAPK (mitogen-activated protein kinase-activated protein kinase)-2-dependent site at Ser^{271} [46,49], an ERK2 (extracellular-signal-regulated kinase 2)-dependent site at Ser^{663} [50], and a cAMP/PKA (protein kinase A)-dependent site at Ser^{523} [51]. The phosphorylation events altering 5-LO have been reviewed previously [46,52,53]. Phosphorylation of the first two sites leads to activation of 5-LO, whereas phosphorylation of Ser^{523} leads to inactivation. This latter pharmacological effect of elevation of cAMP was observed even before knowledge of the 5-LO pathway and the chemical structure of SRS-A. It has been known for some time that β-adrenergic agonists [54], PGE_2 (prostaglandin E_2) [55] or adenosine [56] can elevate cAMP and down-regulate the production of LTC_4.

In certain cells, such as the circulating PMNNs, 5-LO is observed as a cytosolic protein. However, upon adherence of neutrophils [57], 5-LO is found within the nucleus itself. Alveolar macrophages exhibit a nuclear localization of 5-LO that can be reversed upon *ex vivo* culture of these cells [58,59]. Circulating eosinophils have cytosolic 5-LO, but adherence or recruitment drives nuclear 5-LO relocation. There have been reports to suggest that the location of 5-LO either within the cytosol or the nucleus has a profound effect on total leukotriene biosynthetic capability. LTB_4 was found to be synthesized in higher total quantities from adherent neutrophils having nuclear localized 5-LO compared with neutrophils with cytosolic 5-LO [57]. Similar comparative studies were obtained with mast cells and macrophages, leading to the general suggestion that 5-LO, when resident within the nucleus, had a higher total synthetic capacity for leukotrienes [58]. Regardless of the resting cell localization of 5-LO in either cytoplasm or nucleoplasm, cell activation that elevates $[Ca^{2+}]_i$ initiates translocation of 5-LO to the perinuclear envelope.

The location of 5-LO within the nucleus introduces an interesting level of complexity to the 5-LO synthetic cascade, since it is clear that 5-LO must assemble with other proteins, in particular FLAP (5-LO-activating protein), as well as encounter arachidonic acid derived from $cPLA_2\alpha$ (Figure 5). Since the nuclear envelope is a dual-membrane bilayer separated by a luminal space, this has led to speculation that the assembly of the proteins might be different between the nuclear localization on the outer nuclear bilayer relative to the inner nuclear bilayer [60]. Furthermore, the subcellular localization of 5-LO at the cytosolic side of the perinuclear membrane brings into focus

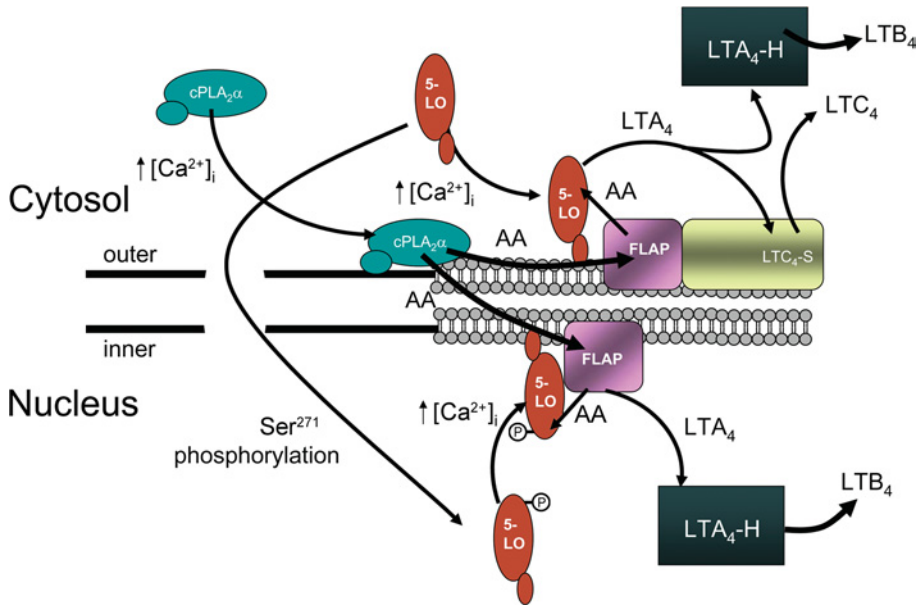

Figure 5 Intracellular organization of the critical enzymes involved in leukotriene biosynthesis

Reorganization includes translocation of cPLA$_2\alpha$ and 5-LO to the perinuclear region, where FLAP and LTC$_4$-S also reside, as a result of elevation of $[Ca^{2+}]_i$. In some cells, 5-LO and LTA$_4$-H are found within the nucleoplasm. In the case of 5-LO, evidence suggests that phosphorylation of Ser^{271} is required for nucleoplasmic location [165]. Elevation of $[Ca^{2+}]_i$ causes translocation to the inner envelope of the dual nuclear bilayer. After conversion of released arachidonatee (AA) into LTA$_4$, LTB$_4$ and LTC$_4$ are then synthesized and exit the cell. An animated version of this Figure can be seen at http://www.BiochemJ.org/bj/405/0379/bj4050379add.htm.

other regulatory mechanisms of 5-LO activity. For example, LOOHs are needed for the initial activation of the catalytic cycle, and peroxidases, including glutathione peroxidase, can reduce 5-LO activity by reducing hydroperoxide tone [61]. During the initial purification of 5-LO, it was observed that in addition to phosphatidylcholine, Ca^{2+} ions and ATP also stimulated 5-LO activity. There appears to be an ATP (nucleotide)-binding site [52] which possibly stabilizes 5-LO, but does not require ATP hydrolysis for this effect. This property of 5-LO was used to first purify 5-LO by binding to an ATP-affinity column.

The production of LTA$_4$ by 5-LO also involves association of additional proteins at the nuclear membrane. The first suggestion of these critical associated proteins came with the identification of a novel protein now called FLAP (Figure 5, and see below). Studies using yeast two-hybrid assays suggested that additional proteins are involved, including human CLP (coactosin-like protein) [62]. Subsequent studies of CLP led to the suggestion that it might be a potential scaffold for 5-LO, on the basis of the profound increase in the quantity of LTA$_4$ made by 5-LO in the presence of this enzyme [63]. Interestingly, CLP is also translocated from the cytosol to the perinuclear region of neutrophils following stimulation.

The expression of 5-LO is typically limited to cells of myeloid origin [64]. This limited expression of 5-LO is thought to be dependent upon 5-LO promoter methylation [65]. 5-LO expression has been found to be induced by TGFβ (transforming growth factor β) and vitamin D$_3$ [66] possibly involving Smads activation [67].

Regulation of leukotriene biosynthesis can also involve transport of LTA$_4$ from the perinuclear region of the synthetic cell, through the cellular plasma membrane, ultimately into a completely different cell containing either LTC$_4$-S or LTA$_4$-H. This is the process of transcellular biosynthesis (see below) and is known to occur rather efficiently between certain cells. However, very little is known about the transport of substrates, including arachidonic acid, specifically within cells such as the human neutrophil that can synthesize leukotrienes. Several proteins are known to stabilize LTA$_4$, including albumin and a family of fatty-acid-binding proteins [68]. An understanding of the role of these interactions and proteins that can stabilize LTA$_4$ as well as the assembly of proteins that constitute a leukotriene-generating machine on the nuclear membrane must await further studies.

Perhaps the most underappreciated mechanism regulating 5-LO activity is the process of self-inactivation. 5-LO was found to be an unstable enzyme during purification studies [69]. Also, hydroperoxides and reactive oxygen products, including the 5-LO products 5-HpETE [70] and LTA$_4$ [71], can inactivate 5-LO through a suicide mechanism. The critical iron ion in the active site could be involved in the irreversible covalent modification reactions due to hydroperoxide-dependent formation of hydroxyl radical [37], but exact details of these chemical events and potential protein-adduct products are unknown.

FLAP

FLAP was discovered in the process of developing inhibitors of 5-LO. The drug candidate, MK886, was found to inhibit leukotriene production in intact cells, but not broken cell preparations or purified 5-LO. After making a photoaffinity labelled analogue, a protein was isolated that bound MK886, but it was not 5-LO [72]. This protein was also found in cells that synthesized leukotrienes, but not in cells lacking 5-LO [73]. This relatively small protein, of 18 kDa, was in fact quite novel in terms of amino acid sequence, but was subsequently found to be related to LTC$_4$-S (31 % overall amino acid identity). Additional members of this family have now been described and have been given the name membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) [74]. FLAP$^{-/-}$ mice have been engineered [75] and have been found to have a phenotype similar to 5-LO$^{-/-}$ mice in

that leukotriene biosynthesis is completely absent. Response to experimental inflammation models is also reduced in FLAP$^{-/-}$ mice [75]. Like 5-LO, FLAP expression can be regulated by TGFβ1 and 1,25-dihydroxyvitamin D_3 as well as other agents in some cells [76,77].

FLAP is an integral membrane protein found to be present at the nuclear envelope [78]. While the three-dimensional structure of FLAP has not been reported, hydropathy plots suggest that it contains three hydrophobic areas separated by two hydrophilic regions [72]. MK886 binds to the first hydrophilic region as determined from mutagenic studies [79].

In spite of the two decades since discovery of FLAP, the exact role played by this protein in the leukotriene synthetic pathway has not been fully elucidated. Several hypotheses have been put forward, including its being a scaffolding protein that facilitates assembly of 5-LO and perhaps cPLA$_2\alpha$ at the nuclear envelope after translocation of these two cytosolic proteins during cellular activation [75,80]. There has been some support for protein–protein interactions as part of the function of FLAP in leukotriene synthesis, but not directly between FLAP and 5-LO. FLAP appears to form dimers in cells [81], and disruption of dimer formation by FLAP inhibitors (e.g. MK886), and even arachidonic acid, reduces LTB_4 production in the stimulated human neutrophil [82]. FLAP and LTC_4 synthase have been found to form a multimeric complex as measured by fluorescence techniques, and these complexes appear to be critical for LTC_4 production in cells expressing both of these proteins [82]. However, MK886 does not alter 5-LO translocation even though LTA_4 production is inhibited. This suggests that the 5-LO–FLAP association is not a major mechanism driving the translocation event [83].

A second hypothesis for the role of FLAP has centred around the property of FLAP as a protein that can bind arachidonic acid and possibly play a role in presenting this substrate to 5-LO [84,85]. Furthermore, arachidonic acid can displace FLAP inhibitors from the FLAP protein, suggesting a binding site for arachidonic acid that is possibly critical for 5-LO activation and enzyme activity. Perhaps the strongest support for the substrate presentation role for FLAP came from studies where Sf9 cells were engineered to express 5-LO or FLAP, or both proteins [85]. The presence of FLAP in those cells expressing 5-LO led to an increased efficiency of LTA_4 production from exogenously added arachidonate, supporting a role in substrate presentation for FLAP. Furthermore, there was a substantial increase in the ability of 5-LO (only in those cells also expressing FLAP) to convert 5-HpETE into LTA_4. An extension of the substrate-binding role for FLAP focuses on 5-HpETE as substrate for 5-LO and the immediate precursor of LTA_4. When translocation of 5-LO to the neutrophil nuclear membrane in an active state was carried out, approx. 50 % of the arachidonic acid metabolized by 5-LO was converted into LTA_4 [86]. This was in contrast with cytosolic 5-LO present in the neutrophils, which could convert less than half of the added arachidonate into LTA_4 products, with the remainder being 5-HpETE. In addition, the membrane-bound 5-LO, which presumably associated with FLAP, appeared to have an altered substrate specificity so that other hydroxyeicosatetraenoic acids (e.g. 12-hydroxyeicosatetraenoic acid or 15-hydroxyeicosatetraenoic acid) were efficiently metabolized by the translocated LO as opposed to cytosolic 5-LO [86].

It is possible that this is a protein partner for 5-LO, critical for the assembly of a machine on the nuclear membrane that can generate leukotrienes. Within the nuclear membrane assembly, FLAP could facilitate the binding of arachidonic acid released from cPLA$_2\alpha$ and enable this arachidonic acid to find the active site of 5-LO. It is also possible that FLAP could protect 5-HpETE, released from 5-LO in the first catalytic cycle of arachidonate oxidation, from cellular peroxidases. FLAP may also facilitate 5-HpETE finding the active site of 5-LO for a second catalytic cycle, yielding LTA_4. In part, this may be due to an altered structure of 5-LO in the nuclear membrane complex so that the translocated 5-LO could accept a bulkier substrate such as 5-HpETE. There is also evidence for the assembly of cPLA$_2$, sPLA$_2$, 5-LO, FLAP and LTC_4-S at the macrophage phagosome built around ingested zymosan particles [87].

LEUKOTRIENE C_4 SYNTHASE

LTC_4-S is the key enzyme in the synthetic pathway of CysLTs in that it catalyses the conjugation of LTA_4 with glutathione to form LTC_4. LTC_4-S is an 18 kDa protein that is expressed in cells of myeloid origin such as basophils, eosinophils, mast cells and monocytes/macrophages, which also express 5-LO [88]. It is also expressed in platelets, which do not contain 5-LO and cannot synthesize LTA_4 on their own [89]. However, platelets are able to use LTA_4 released by 5-LO-containing cells and synthesize LTC_4 through transcellular metabolism [90]. As mentioned above, LTC_4-S exhibits approx. 31 % amino acid identity with FLAP, and is inhibited by the FLAP inhibitor MK886 [91]. Both proteins are members of the MAPEG family [74], which also includes mGST (microsomal glutathione S-transferase)-1, -2 and -3 and mPGES-1 (microsomal PGE synthase-1).

LTC_4-S is an integral membrane protein that is present on the endoplasmic reticulum and on the cytoplasmic leaflet of the nuclear envelope. It contains four transmembrane domains, and has been shown to form oligomers, as well as hetero-oligomers with FLAP. The structure of two-dimensional crystals of mGST-1 [92] suggested that this enzyme also forms trimers. On the basis of mutagenesis studies, LTC_4-S has been shown to exist at the nuclear membrane as a non-covalent dimer [88,93]. Additional mutagenesis studies have also identified two critical residues in the active site supporting a catalytic mechanism. Arg^{51} acts as an acid catalyst for the opening of the epoxide ring in the LTA_4 substrate, whereas Tyr^{93} is a base catalyst for the generation of the thiolate anion of the glutathione substrate [93]. Each of these residues is localized on one of the two hydrophilic loops of LTC_4-S, which are oriented to the same side of the membrane, probably the luminal space of the outer nuclear envelope and the endoplasmic reticulum. The evidence supporting that LTC_4 is synthesized in the lumen suggests that an additional step is necessary to transport this eicosanoid to the cytoplasm, from where it can be secreted via the glutathione adduct transporter, MRP-1 (multidrug-resistance protein-1) [94].

Enzymes other than LTC_4-S are able to catalyse the synthesis of LTC_4 from LTA_4 and glutathione. mGST-2 is responsible for the ability of endothelial cells to produce LTC_4 through transcellular metabolism of LTA_4 [95,96]. However, studies with transgenic mice deficient in LTC_4-S demonstrated that this enzyme was the major source of LTC_4 in most tissues, with the exception of testis, probably reflecting the role of mGST-2 or mGST-3 in this organ [97]. LTC_4-S-deficient mice are healthy and fertile, but they exhibit reduced inflammatory responses, including zymosan-induced plasma extravasation, IgE-mediated passive anaphylaxis and antigen-induced pulmonary inflammation. These data highlight the relevance of CysLTs and of LTC_4-S in inflammation. Clinical data from several human subjects with a deficiency in LTC_4-S show a strong correlation between undetectable CysLTs in cerebrospinal fluid and mental retardation, suggesting that a genetic metabolic error in CysLT synthesis may be responsible for the phenotype [98], although more data will be needed to ascertain

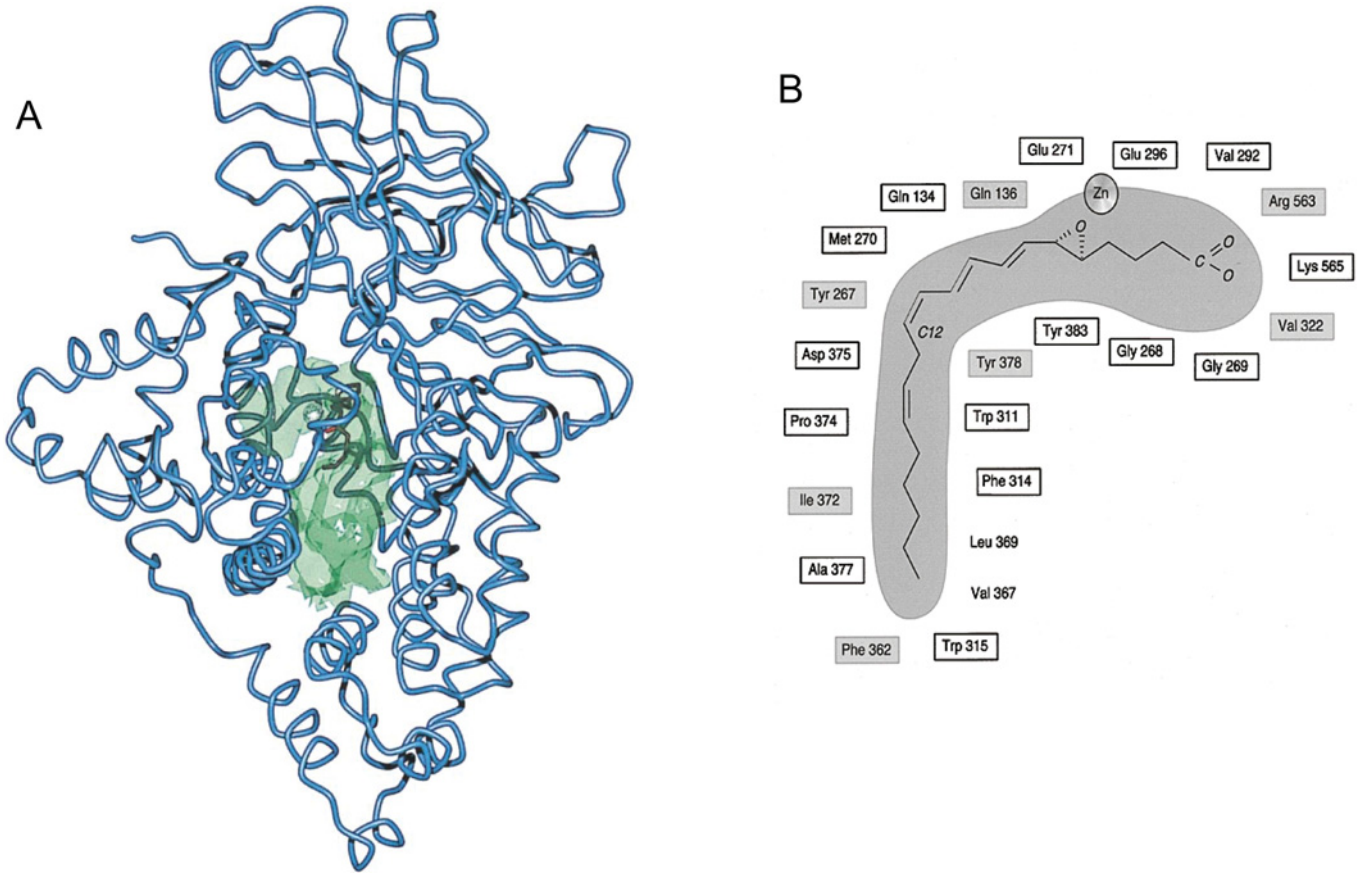

Figure 6 Structure and active site model of LTA_4-H

(**A**) Three-dimensional structure of LTA_4-H with a semi-transparent surface representation of the active site containing bestatin. (**B**) Schematic drawing of the proposed active site of LTA_4-H indicating Zn^{2+} and LTA_4 binding with conserved amino acid residues in the boxes. Reprinted by permission from Macmillan Publishers Ltd: Nature Structural Biology [107], © 2001.

this possibility or the role of LTC_4-S in the central nervous system development.

LEUKOTRIENE A_4 HYDROLASE

LTA_4-H catalyses the conversion of LTA_4 into the dihydroxy leukotriene, LTB_4. As a result of the concerted effort of Haeggstrom [99], LTA_4-H is the best understood enzyme in the leukotriene cascade from its three-dimensional structure to catalytic mechanism, and even molecular evolution.

LTA_4-H is a 70 kDa zinc-containing metalloprotein found in the cytosol of cells [100]. There have been reports that LTA_4-H can be localized within the nucleus in some cells [101]. This enzyme, formally an epoxide hydrolase in that it catalyses the addition of water to an epoxide moiety, has no resemblance to the epoxide hydrolases that act on drug epoxide metabolites [102]. When the amino acid sequence of LTA_4-H was determined [103], the similarity of this enzyme to known zinc-containing aminopeptidases was recognized [104]. This led to the discovery of a second activity of LTA_4-H as an aminopeptidase [105] and recognition of the possible evolution of this enzyme from a progenitor aminopeptidase [106]. Inhibitors of this peptidase activity, such as bestatin, were also found to inhibit the conversion of LTA_4 into LTB_4 [105], suggesting the involvement of the same active site which contains a catalytic zinc atom for both the peptidase and epoxide hydrolase enzymatic mechanisms.

The competitive inhibitor bestatin was employed in the crystallographic analysis of the three-dimensional structure of LTA_4-H at 1.95 Å resolution, which revealed three domains with a catalytic domain very similar to that of thermolysin [107]. More recently, the X-ray structure of LTA_4-H was determined complexed with captopril [108]. Using X-ray structures, LTA_4 has been modelled into the proposed active site which has an L-shaped hydrophobic groove between the three domains of LTA_4-H. This groove is only 6–7 Å wide, but quite nicely accepts the structure of the hydrophobic tail of LTA_4, which forces the triene epoxide to adopt a conformation with a 'pro-*cis*' orientation of the Δ^6 double bond of LTA_4 in the active site (Figure 6). The critical involvement of numerous amino acid residues is now understood in terms of holding the flexible LTA_4 in the proper orientation. It has been reasoned that the epoxide ring can be opened by the co-ordinated involvement of the Zn^{2+}, water and Glu^{271} to form a delocalized carbocation over C-6 to C-12 of LTA_4 [99]. The stereospecific addition of water to form the 12(*R*) hydroxy group of LTB_4 appears to be carried out by base catalysis of Asp^{375} acting on water in the appropriate position of the LTA_4 chain to add the hydroxyl anion to C-12 in correct orientation.

Regulation of LTA_4-H activity is not as complex as that of 5-LO, but it is known to be affected by suicide inactivation. In this reaction, Tyr^{378} can covalently react with LTA_4 as a part of the catalytic mechanism of LTB_4 formation [109]. When LTA_3 was the substrate of LTA_4-H, it appeared that Tyr^{383} covalently adducted [110]. Both of these tyrosine side chains appear very

close to the active site (Figure 6). Phosphorylation of LTA_4-H has been reported (at Ser^{415}) [99], but there is little evidence at present to suggest that LTA_4-H activity is regulated by protein kinases.

LTA_4-H is widely expressed in numerous cells and tissues, and clearly it is not restricted to those cells that express 5-LO [111]. Surprisingly, even human red blood cells were found to contain LTA_4-H in the cytosol [112], which was one of the initial findings that led to the formulation of the transcellular biosynthesis hypothesis for leukotrienes. Although studies with knockout mice demonstrated the key role of LTA_4-H in LTB_4 production and inflammation-induced PMNN influx, the role of the aminopeptidase activity of LTA_4-H remains unknown [113].

LEUKOTRIENE METABOLISM

Molecules which function as signals to co-ordinate biochemical events between cells must be subject to a mechanism that terminates their activity. Leukotrienes are inactivated by metabolic conversion into a number of products that are not recognized by specific receptors and into derivatives that facilitate elimination. Since LTB_4 is essentially a hydroxy fatty acid, it is perhaps not surprising that fatty acid degradation pathways such as β-oxidation and conjugation reactions with glucuronic acid are involved. There are also specific enzymes, including unique cytochrome P450s and oxidoreductases that metabolize LTB_4. LTC_4, being a peptidolipid, is a substrate for peptidases that form LTE_4 as the major metabolite. LTE_4 is then processed by ω-oxidation, followed by β-oxidation.

ω-Oxidation

Shortly after the discovery of LTB_4, the neutrophil was found to metabolize this eicosanoid into 20-hydroxy-LTB_4 (20-OH-LTB_4) and 20-carboxy-LTB_4 (20-COOH-LTB_4) (Figure 7) [114]. Subsequent studies revealed that a novel cytochrome P450 efficiently carried out this methyl terminal oxidation [115]. This enzyme was found to be a member of a larger series of related P450s termed CYP4F, many of which have now been cloned and expressed [116,117]. Over 14 different members are now known throughout the animal kingdom, including five human CYP4F genes [118]. These enzymes can be induced by various agents, for example the human neutrophil LTB_4-ω-hydroxylase (CYP4F3A) can be induced by retinoic acid and phorbol ester [118].

Interestingly 20-OH-LTB_4 binds quite well to the BLT-1 receptor (K_i of 0.54 nM compared with 0.7 nM for LTB_4) but not to the BLT-2 receptor (K_i of 41 nM compared with 2.3 nM for LTB_4) [119]. Thus ω-oxidation itself does not inactivate LTB_4, certainly for chemotaxis of neutrophils. However, 20-COOH-LTB_4 is an inactive metabolite. Further oxidation of the ω-hydroxy moiety to 20-COOH-LTB_4 by the neutrophil can be catalysed by CYP4F3A, which was shown to proceed in two steps via the 20-oxo-LTB_4 intermediate [120]. In other tissues, such as the liver, an alternative pathway for subsequent oxidation of 20-OH-LTB_4 was found to involve ADH (alcohol dehydrogenase) and AldDH (aldehyde dehydrogenase). Evidence for participation of these enzymes came from the inhibition of 20-OH-LTB_4 oxidation by liver microsomes when ethanol was present at concentrations relevant to human consumption [121]. Ethanol can also alter the LTB_4 metabolic pathway found in the liver with formation of two biologically active metabolites, 3-hydroxy-LTB_4 and 3,20-hydroxy-LTB_4 [122]. This alteration in the formation of hepatic metabolites supports the role of ω-oxidation to 20-COOH-LTB_4 as a necessary intermediate step for subsequent β-oxidation, a step that has been termed 'activation' of the metabolic pathway for LTB_4.

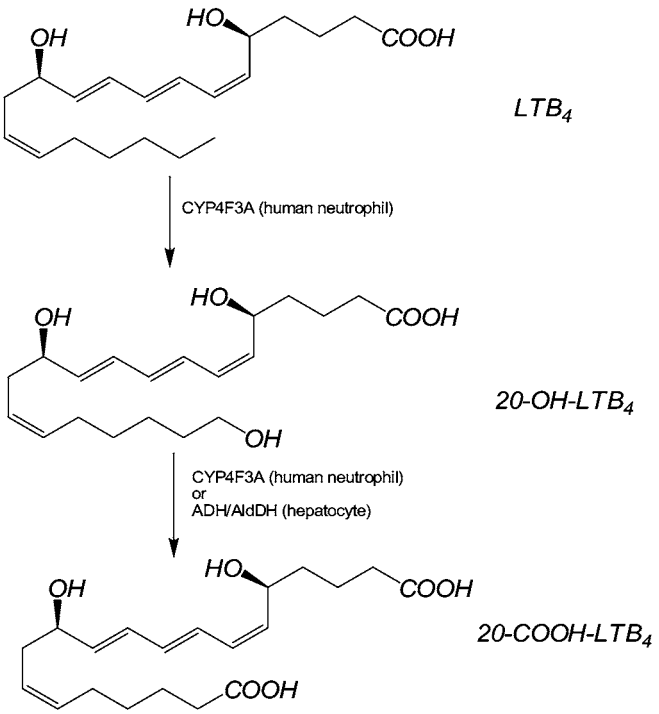

Figure 7 Pathway of metabolism of LTB_4 by ω-oxidation

The pathway is catalysed by CYP4F3A in the human neutrophil to form 20-OH-LTB_4, which can be oxidized further either by the P450 enzyme or by ADH and AldDH to form 20-COOH-LTB_4.

Measurement of urinary metabolites of LTB_4 has not been possible in normal human subjects, which has made it difficult to study normal LTB_4 biosynthesis *in vivo*. Several possible reasons for this include the fact that LTB_4 is a potent lipid mediator and is therefore not synthesized in large amounts; secondly, a large number of metabolites are derived from LTB_4; and thirdly, the overall extent of conversion of LTB_4 into acetyl-CoA by several cycles of β-oxidation is likely to be quite high. Recent studies of patients with Sjögren–Larson syndrome who have a mutation in the gene that encodes FALDH (fatty aldehyde dehydrogenase), reported high levels of urinary LTB_4 and 20-OH-LTB_4 [123]. This observation supports pre-eminence of the ADH/AldDH pathway for ω-oxidation of 20-OH-LTB_4 from endogenously generated LTB_4. Another genetic disorder, Zellweger syndrome, in which there is a deficiency in peroxisomal β-oxidation, is another condition in which high urinary levels of LTB_4 and 20-COOH-LTB_4 have been observed [124].

Studies of LTB_4 metabolism by various CYP4F isoforms *in vitro* revealed formation of several unexpected products, namely ω-1, ω-2 and ω-3 metabolites. The importance of these pathways was supported by characterization of urinary metabolites of LTB_4 [125]. Although ω-hydroxylation is the only CYP4F3A product of LTB_4 metabolism, CYP4F6 was found to form both 19-OH- and 18-OH-LTB_4 [126]. Rodent neutrophils (which express CYP4F18) were found to convert LTB_4 into 19-OH-LTB_4, with only modest ω-2 oxidizing activity to yield 18-OH-LTB_4 [127]. There is no known biological activity of these metabolites and very little known about subsequent disposition. An 18-oxo metabolite has been reported [127], as well as glucuronides [125]. This latter metabolic transformation was only recognized fairly recently as a major pathway for LTB_4 metabolism in human subjects when a large quantity of LTB_4 (50 μg/kg) was injected into several human subjects as part of a Phase I

clinical trial of LTB_4 as an immunomodulator [125]. When urinary metabolites were characterized structurally, one of the metabolites was identified as 17-OH-LTB_4 after treatment of the urine with β-glucuronidase. This suggested that the 17-OH-LTB_4-glucuronide may be one of the more abundant metabolites excreted into urine. These alternative ω-oxidized metabolites are probably removed from mitochondrial or peroxisomal β-oxidation cycles because of reduced substrate affinity for CoA ester formation at the C-1 carboxy moiety, which leaves the glucuronidation pathway available for conjugation reactions before elimination.

Methyl terminal ω-oxidation of LTE_4 is a major metabolic process [128], but no studies have described the specific P450 isoenzymes mediating this process. Intact LTE_4 is excreted into urine as a major metabolite, accounting for 4.3 ± 0.9 % total LTC_4 production in human subjects [129]. This is a sufficiently abundant metabolite so that sensitive and specific assays can be used to measure LTC_4 biosynthesis *in vivo*. Interestingly, the metabolites derived from LTE_4 ω/β-oxidation are excreted at similar levels, but only a few reports have appeared to measure these excreted metabolites [130].

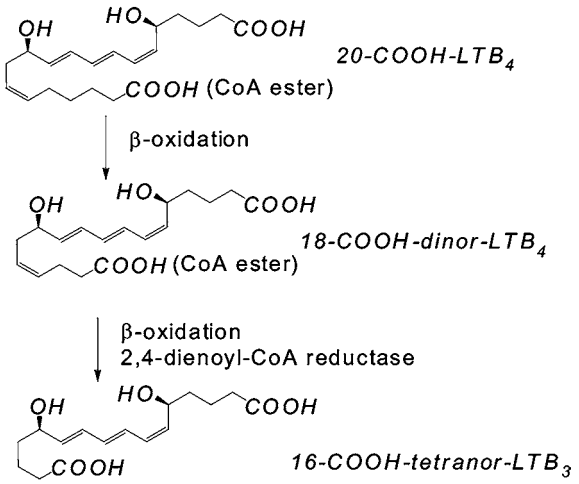

Figure 8 β-Oxidation of 20-COOH-LTB_4 as a CoA ester leading to 18-COOH-dinor-LTB_4 and 16-COOH-tetranor-LTB_3 which have been found as urinary metabolites

β-Oxidation

The major metabolites of eicosanoids are products of mitochondrial and peroxisomal β-oxidation formed by the actions of enzymes that also chain-shorten naturally occurring saturated and polyunsaturated fatty acids from the corresponding carboxyl-CoA ester derivatives. A major difference between prostaglandin and leukotriene metabolism is that prostaglandins are chain-shortened from the C-1 carboxy group. In contrast, leukotrienes require initial ω-oxidation to form ω-COOH-LTB_4 or ω-COOH-LTE_4, and CoA ester formation proceeds from the newly formed ω-carboxy group. The biochemical mechanism for this has not been investigated, but perhaps relates to the affinity of both of these carboxylic acids for the enzymes responsible for CoA ester synthesis. Also, leukotrienes have a C-5 hydroxy group as opposed to prostaglandins, which have a double bond at C-5. Formation of CoA esters of ω-COOH leukotrienes has not been studied in detail, even though these derivatives are the critical intermediates for β-oxidation. Both 20-COOH-LTB_4 and 20-COOH-LTE_4 are metabolized by peroxisomal β-oxidation pathway, but only 20-COOH-LTB_4 is metabolized within the mitochondrion by β-oxidation [131].

The steps of chemical transformation that take place in peroxisomes start with 20-COOH-CoA esters which are β-oxidized to a Δ^{18}-intermediate, catalysed by acyl-CoA oxidase (Figure 8). This is followed by hydration of the C-18 double bond, then oxidation to form the 18-oxo-CoA ester by the peroxisomal bifunctional enzyme having both CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity. The penultimate carbon atoms of leukotrienes are then removed by 3-oxoacyl CoA thiolase, yielding the CoA ester of the 18-COOH-LTB_4 or 18-COOH-LTE_4. This process is repeated for a second round of β-oxidation. There is a complication in that insertion of a new double bond adjacent to the 18-carboxy-CoA ester results in a conjugated diene involving the double bond at carbon atoms 14–15 in both LTB_4 and LTE_4. The peroxisomal enzyme 2,4-dieneoyl-CoA reductase reduces this diene to a monoene at carbon-16. This multifunctional enzyme also has Δ^3,Δ^2 acyl-CoA isomerase activity [132] which moves the Δ^{16} double bond to Δ^{17} required for subsequent β-oxidation to proceed. After removal of the two terminal carbon atoms as acetyl-CoA, the metabolites 16-COOH-tetranor-LTB_3 and 16-COOH-tetranor-LTE_3 result. The major metabolite of LTB_4 incubated without hepatocytes was found to be 18-COOH-LTB_4 [133] and this metabolite was also a significant metabolite of LTB_4 present in human urine [125].

LTE_4 is metabolized (Figure 9) by similar pathways in rat hepatocytes and in human subjects [128]. After initial ω-oxidation to 20-COOH-LTE_4 and formation of the 20-CoA ester, metabolism proceeds through at least three stages of β-oxidation exclusively within the peroxisome [130,131]. In rat hepatocytes, the most abundant metabolite observed was *N*-acetyl-16-COOH-tetranor-LTE_3. N-acetylation is a major metabolic step for LTE_4 in the rat even before ω/β-oxidation, but it is not a major metabolic pathway for LTE_4 in human tissues [128].

12-Hydroxydehydrogenase/15-oxo-prostaglandin-13-reductase (12HDH/15oPGR)

The 12HDH/15oPGR pathway (Figure 10) is fairly specific for LTB_4 metabolism in human and porcine tissues, and this enzyme appears to recognize the structural motif [R-CH(OH)-(*trans*)-CH = CH-R′]. It was first discovered in studies of LTB_4 metabolism in porcine leucocytes, where several metabolites were found that did not retain the conjugated triene structure typical for leukotrienes [134]. This complex pathway had three separate structural conversions of the starting substrate. The first step oxidizes the 12(*R*) hydroxy group of LTB_4 into the 12-oxo moiety. This step is catalysed by 12HDH/15oPGR. A detailed understanding of the mechanism of this oxidation step has been suggested following elucidation of the X-ray crystallographic structure of guinea-pig 12HDH/15oPGR [135].

The product of 12-hydroxy-oxidation is a conjugated ketone with a structural motif [R-CO-(*trans*)-CH = CH-R′] common to many leukotriene and prostaglandin metabolites and as such is a structural unit that can be reduced in two steps to [R-CH(OH)-CH_2CH_2-R′]. 12HDH/15oPGR is a bifunctional enzyme which can carry out reduction of 15-oxo prostaglandins into 15-oxo-13,14-dihydroprostaglandin metabolites. However it is not clear at this time whether the human or porcine enzyme can reduce 12-oxo-LTB_4 to 12-oxo-10,11-dihydro-LTB_4 or whether a separate reductase carries out this step [136].

Human keratinocytes were found to extensively metabolize LTB_4 by this 12HDH/15oPGR pathway [137]. Interestingly, a rather unusual metabolite, 10-HOTrE (10-hydroxyoctadecatrienoic acid), was observed in these studies, which suggested that a

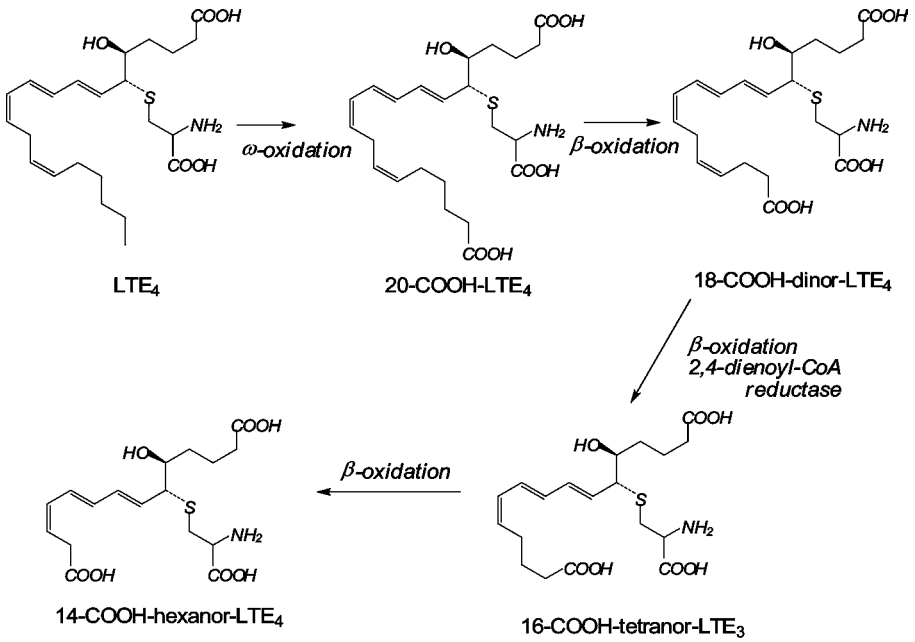

Figure 9 Pathway of the ω- and subsequent β-oxidation of LTE_4 showing the chemical structure of metabolites identified in the urine of human subjects

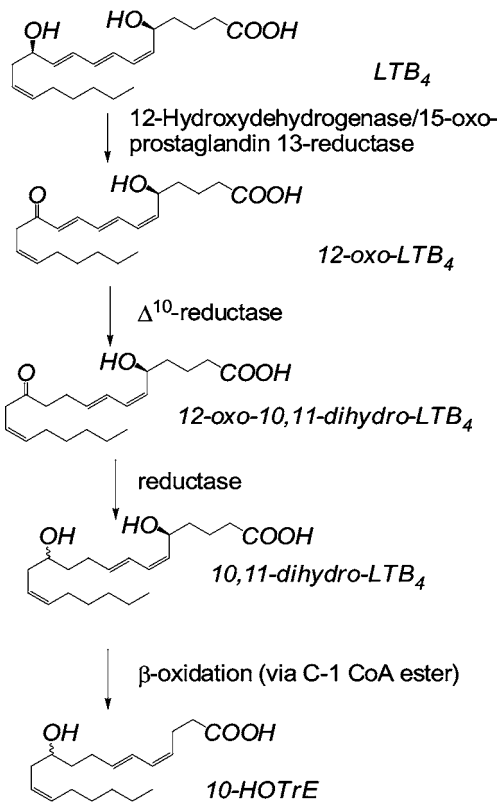

Figure 10 Metabolism of LTB_4 by the 12HDH/15oPGR pathway leading to the reduced metabolite 10,11-dihydroxy-LTB_4 which was found to be β-oxidized after C-1 CoA ester formation ultimately forming 10-HOTrE

C-1 CoA ester can be formed from 10,11-dihydro-LTB_4. Consistent with this possibility was the appearance of a chain-elongated metabolite of 10,11-dihydro-LTB_4 that would also proceed from the C-1 CoA ester. In addition, there appeared to be very low CYP4F activity present in the human keratinocytes, since very few of the observed metabolites were ω-oxidized. In the studies of LTB_4 metabolism in human subjects [125], numerous metabolites appeared to be generated by this 12-hydroxydehydrogenase pathway including 10,11-dihydro-20- and 18-COOH-LTB_4 as well as the glucuronide conjugate of 10-HOTrE.

This same structural unit [R-CH(OH)-(*trans*)-CH = CH-R′] is found in the 6-*trans*-LTB_4 isomers formed by non-enzymatic hydrolysis of LTA_4. The 6-*trans*-LTB_4 and 6-*trans*-12-epi-LTB_4 have low affinity for the CYP4F3A present in the human neutrophil which catalyses ω-oxidation of LTB_4. However, human neutrophils do have an enzymatic capacity to oxidize the 6-*trans*-LTB_4 isomers into 5-oxo-Δ^6-*trans*-LTB_4 isomers that were found to be reduced further to the motifs [R-CO-CH_2CH_2-R′] and [R-CH(OH)-CH_2CH_2-R′] [138]. Such a transformation is strikingly similar to the pathway described above for the 12HDH/15oPGR pathway. Whether a second protein is responsible for these metabolic transformations needs further study, but these metabolites reveal the importance of the geometry of the double bond allylic to a hydroxy group in directing metabolic disposition of leukotrienes.

CysLT peptide hydrolysis

Metabolism of LTC_4 proceeds initially by peptide cleavage reactions typical of glutathione adducts. Since LTC_4, LTD_4 and LTE_4 interact with different binding affinity to both $CysLT_1$ and $CysLT_2$, this metabolic pathway plays a central role in altering biological responses to the initiation of LTC_4 biosynthesis at the tissue site. The peptide bonds involved are the unusual γ-glutamyl peptide bond to the N-terminus of cysteine and the glycinyl amino group forming the amide with the cysteinyl carboxy group. The observed order of peptide hydrolysis in biological systems proceeds initially from LTC_4 to LTD_4 with cleavage of the γ-glutamic acid amide bond. Another CysLT has been reported and named LTF_4, which retains the γ-glutamic acid in the CysLT structure but no glycine [139], but this metabolite has not been observed either *in vitro* or *in vivo* in systems that generate LTC_4.

The importance of GGT (γ-glutamyl transpeptidase) in this LTC_4 metabolism pathway was recognized quite early after the discovery of LTC_4, in that LTC_4 was found to be a good substrate for GGT isolated from various tissues [140]. Even human plasma was reported to have one or more GGT activities that could convert LTC_4 into LTD_4. GGT is a heterodimeric glycosylated enzyme located on the external side of the plasma membrane of cells [141,142].

In studies of human GGT genes, a related gene was found which when expressed encoded for a protein (termed GGT-rel) that had only 40 % amino acid similarity to human GGT, but efficiently converted LTC_4 into LTD_4 [143]. This finding raised the possibility of a specific GGT isoenzyme that metabolized LTC_4 *in vivo*. Support for this hypothesis came from studies of GGT-knockout mice which were found to have substantial capacity to metabolize LTC_4 to LTD_4 [144]. A protein responsible for this residual peptidase activity was isolated from the $GGT^{-/-}$ mice, partially purified and found to cleave LTC_4, but not the normal GGT substrates used for biochemical assays [144]. This protein, termed γ-glutamyl leukotrienase (GGL) [145], was similar to GGT and was thus considered to be a member of the GGT family. $GGL^{-/-}$ mice were found to accumulate LTC_4, but it took a double-mutant mouse, deficient in both GGT and GGL, to prevent conversion of all LTC_4 into LTD_4 [146]. In mice, this unique GGT family member, GGL, was found to be expressed on endothelial cells. It is thought to play a central role in catalysing the initial peptide cleavage of LTC_4 to LTD_4 [147].

Previously, several human subjects that were deficient in GGT were studied in terms of their ability to metabolize CysLTs [148]. In these subjects, CysLTs excreted into urine were analysed as well as the capacity of circulating monocytes to synthesize CysLTs after stimulation with A23187 *in vitro* [148]. In the GGT-deficient human subjects, LTC_4 could be readily detected in the urine, but not LTD_4 or LTE_4. This is in sharp contrast with normal subjects where no LTD_4 or LTC_4 has ever been observed excreted into urine, whereas LTE_4 is readily measured in urine or in bile [128,130]. Stimulating monocytes from normal human subjects resulted in equal quantities of LTC_4, LTD_4 and LTE_4, but the GGT-deficient monocytes could produce only LTC_4. These results suggested that GGT plays a central role in converting LTC_4 into LTD_4 in humans, although the exact gene defect in these deficient human subjects was not described in detail. Whether or not GGT-rel was deficient as well in these patients was not described.

Studies investigating the effect of dexamethasone on altering metabolism of LTC_4 revealed that bronchoepithelial cells (16-HBE cells) significantly increased LTC_4 metabolism even at doses of 0.1–1 μM dexamethasone [149]. Furthermore, at these concentrations, there was an increase in mRNA for GGT-rel, but not GGT, suggesting a role for GGT-rel in the metabolism of LTC_4 to LTD_4. This was reported as a novel mechanism of action for glucocorticoid steroids on bronchoepithelial cells in modulating LTC_4 metabolism. Certainly, additional studies are needed to resolve the importance of GGT and GGT-rel for the metabolism of LTC_4 in humans.

The conversion of LTD_4 into LTE_4 involves hydrolysis of the Cys–Gly amide bond which can be catalysed by a number of dipeptidases *in vitro*. This hydrolysis leads to alteration of biological activity [150], as well as the affinity for $CysLT_1$ and $CysLT_2$ [10]. Leucine aminopeptidase was found to have this activity shortly after the structural characterization of LTC_4 and LTD_4 [151]. It was quite reasonable to test the activity of leucine aminopeptidase because of the role of this enzyme in the metabolism of glutathione. However, recent studies have measured a fairly low activity of leucine aminopeptidase towards hydrolysis of LTD_4 [152].

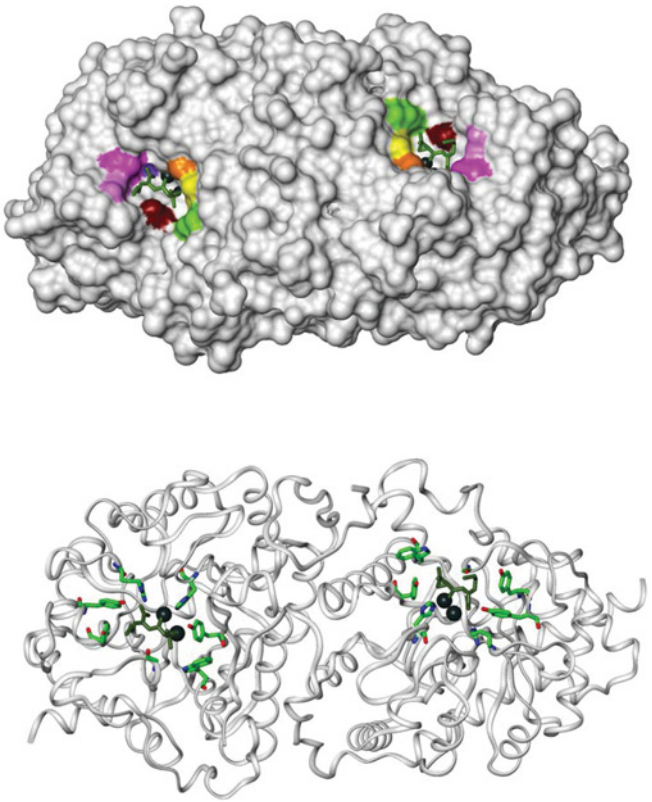

Figure 11 Three-dimensional structure of microsomal MBD as a dimer, determined by X-ray crystallography (PDB code 1ITU) having the inhibitor cilastatin in the active site [161]

The catalytic site residues are coloured in the Figure. Cilastatin is shown as a stick model in the active cavities. (**A**) Surface representation of the dimer. (**B**) Ribbon representation of the backbone to reveal the Zn^{2+} (black spheres) in the active site. These structures were prepared using Insight-II by Dr Tatiana Kutateladze.

As a result of studies directed at characterization of proteins that could specifically hydrolyse the Cys–Gly amide bonds, a membrane-associated zinc metalloprotein dipeptidase was isolated from rat liver [153]. Upon examination of substrate specificity, this dipeptidase was found to have the capacity to convert LTD_4 into LTE_4. Subcellular localization studies of the enzymatic activity that could convert LTD_4 into LTE_4 also led to the description of membrane or microsomal proteins from human neutrophils [154], and rat neutrophils, lymphocytes and macrophages [155]. The membrane fraction of sheep lungs was also found to contain LTD_4 dipeptidase activity [156]. These studies have clearly focused attention on a membrane-bound dipeptidase as being responsible for metabolism of LTD_4 *in vivo*.

A specific membrane-associated dipeptidase called microsomal MBD (membrane-bound dipeptidase) was purified from human kidneys and was found to have considerable activity in metabolizing LTD_4 to LTE_4, suggesting that it might play a central role in CysLT metabolism [157]. Lung dipeptidase isolated from sheep [156] was subsequently cloned and was found to have very similar homology with this kidney dipeptidase [158], further supporting MBD as an important protein in mediating LTD_4 inactivation. This MBD was deleted in mice, but the $MBD^{-/-}$ mice still retained considerable capacity to convert LTD_4 into LTE_4 [159]. Some tissues, such as the small intestine, retained 80–90 % of their activity to hydrolyse LTD_4 compared with wild-type, whereas lung and heart LTD_4 metabolizing activity was reduced to 40 %. These results suggested that MBD was partially responsible for LTD_4

metabolism, but, more importantly, that several different peptidases might be responsible for this activity *in vivo*. When a gene database was searched for genes related to MBD, two additional genes were discovered [160]. After expression of the gene termed MBD-2, the protein was found to have 33 % identity with mouse MBD-1 and, furthermore, was capable of metabolizing LTD_4 to LTE_4 with a very similar activity to that of MBD-1. The second protein, termed MBD-3, did not have any capacity to metabolize LTD_4.

MBD is a zinc metalloprotein (Figure 11) with a unique zinc-binding motif [161]. It is an active enzyme that is linked to the plasma membrane by a glycosylphosphatidylinositol anchor. It can hydrolyse various amides through its dipeptidase activity, including certain antibiotics. Furthermore, a high-affinity reversible inhibitor called cilastatin has been found [162]. Although there is strong evidence for MBD, and now MBD-2, as having an important role in the metabolic inactivation of LTD_4, further studies will undoubtedly reveal if these are the only two dipeptidases which are responsible for this critical termination of biological activity of CysLTs.

CONCLUSION

As a result of extensive work over the past three decades, a fair understanding of the biosynthesis and metabolism of leukotrienes has emerged. Surprising features of this cascade include the recognition of the assembly of critical enzymes at the perinuclear region of the cell and even localization of 5-LO within the nucleus of some cells. Under some situations, the budding phagosome has been found to assemble these proteins. Non-enzymatic proteins such as FLAP are now known as critical partners of this protein-machine assembly. An unexpected pathway of leukotriene biosynthesis involves the transfer of the chemically reactive intermediate, LTA_4, from the biosynthetic cell followed by conversion into LTB_4 or LTC_4 by other cells that do not express 5-LO. Also, a deeper understanding of the physiological role for leukotrienes is emerging in studies of human metabolic defects that interfere with the rapid metabolism of LTB_4 and LTC_4. Yet, several fundamental questions remain.

The recent observation that profound stimulation of leukotriene biosynthesis can result from inhibition of lysophospholipid acyltransferase suggest that reacylation of arachidonic acid regulates leukotriene biosynthesis in certain cells. Nothing is known about this family of enzymes and whether or not they are specific for arachidonate reacylation. 5-LO has yet to be structurally characterized at the three-dimensional level, and this has hampered a full understanding of the catalytic mechanisms that leads to the production of 5-HpETE and its conversion into LTA_4. Furthermore, the translocation of 5-LO to the perinuclear membrane is only poorly understood in terms of proteins that become associated at this site to form the machine that produces LTA_4. This lack of information is highlighted by our lack of a complete understanding of the role of FLAP in this system. Future studies will hopefully reveal the role of FLAP in mechanistic detail along with other associated proteins.

The movement of LTA_4 within the cell is also poorly understood. The chemical reactivity of LTA_4 requires engagement of proteins that can sequester this lipid from water, yet permit transfer to the active site of cytosolic (or nucleoplasmic) LTA_4-H or LTC_4-S. Even less understood is the exact mechanism and protein involvement in transcellular biosynthesis where LTA_4 leaves the perinuclear region and is transported into another cell. While this process has been suggested to involve transport proteins, an understanding of the role of cell–cell contact and acceptor cell LTA_4-stabilization mechanisms are lacking. The spectrum of peptidases involved in CysLT metabolism have yet to be fully elucidated along with an understanding of those enzymes which can ω-oxidize LTB_4 and CysLTs. Finally, studies of leukotriene metabolism have revealed an important role of this class of lipid mediators in organism development, particularly from the standpoint of leukotriene activity in the face of metabolic enzymatic defects that become associated with mental retardation. These observations, however, do expand our understanding of the importance of this arachidonic acid cascade in normal physiology as well as pathophysiology.

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