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Eicosanoid biosynthesis in marine mammals

Florian Reisch, Kumar Reddy Kakularam, Sabine Stehling, Dagmar Heydeck and Hartmut Kuhn

Institute of Biochemistry, Charité – University Medicine Berlin, Corporate member of Free University Berlin, Humboldt University Berlin and Berlin Institute of Health, Berlin, Germany

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Correspondence
H. Kuhn, Institute of Biochemistry (CC2), Charité - University Medicine Berlin, Charitéplatz 1, 10117 Berlin, Germany
Email: hartmut.kuehn@charite.de

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After 300 million years of evolution, the first land-living mammals reentered the marine environment some 50 million years ago. The driving forces for this dramatic lifestyle change are still a matter of discussion but the struggle for food resources and the opportunity to escape predators probably contributed. Reentering the oceans requires metabolic adaption putting evolutionary pressure on a number of genes. To explore whether eicosanoid signaling has been part of this adaptive response, we first explored whether the genomes of marine mammals involve functional genes encoding for key enzymes of eicosanoid biosynthesis. Cyclooxygenase (COX) and lipoxygenase (ALOX) genes are present in the genome of all marine mammals tested. Interestingly, ALOX12B, which has been implicated in skin development of land-living mammals, is lacking in whales and dolphins and genes encoding for its sister enzyme (ALOXE3) involve premature stop codons and/or frameshifting point mutations, which interrupt the open reading frames. ALOX15 orthologs have been detected in all marine mammals, and the recombinant enzymes exhibit similar catalytic properties as those of land-living species. All marine mammals express arachidonic acid 12-lipoxygenating ALOX15 orthologs, and these data are consistent with the Evolutionary Hypothesis of ALOX15 specificity. These enzymes exhibit membrane oxygenase activity and introduction of big amino acids at the triad positions altered the reaction specificity in favor of arachidonic acid 15-lipoxygenation. Thus, the ALOX15 orthologs of marine mammals follow the Triad concept explaining their catalytic specificity.

Introduction

Marine mammals form a diverse group of mammalian species that heavily rely on aquatic environments for their existence [1,2]. These animals do not represent a distinct taxon, and some marine mammalian species are more closely related to land-living relatives from the evolutionary point of view [3–5]. The most important classifying parameter for marine mammals is their reliance on the marine environment for feeding [6]. According to their evolutionary relatedness, more than 100 marine mammalian species are classified into five different groups: (i) whales and dolphins (*Cetaceans*), (ii) sea cows (*Sirenians*), (iii) seals, sea lions,

Abbreviations
11-H(p)ETE, 11-hydro(pero)xy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid; 12-H(p)ETE, 12-hydro(pero)xy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid; 13-H(p)ODE, 13-hydro(pero)xy-9*Z*,11*E*-octadecadienoic acid; 15-H(p)ETE, 15-hydro(pero)xy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid; 5-HpETE, 5-hydro(pero)xy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; 7-HpETE, 7-hydro(pero)xy-5*Z*,8*Z*,11*Z*,14Z-eicosatetraenoic acid; 8-HETE, 8-hydro(pero)xy-5*Z*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid; 9-H(p)ETE, 9-hydro(pero)xy-5*Z*,7*E*,11*Z*,14*Z*-eicosatetraenoic acid; 9-H(p)ODE, 9-hydro(pero)xy-10E,12Z-octadecadienoic acid; ALOX, arachidonic acid lipoxygenase; ALOX12, arachidonic acid lipoxygenase 12 (platelet-type 12-LOX); ALOX12B, arachidonic acid lipoxygenase 12B (12R-LOX); ALOX15, arachidonic acid lipoxygenase 15 (12/15-LOX); ALOX15B, arachidonic acid lipoxygenase 15B (15-LOX2); ALOX5, arachidonic acid lipoxygenase 5 (5-LOX); ALOXE3, epidermal arachidonic acid lipoxygenase 3 (epidermal LOX3); COX, cyclooxygenase; CP-HPLC, chiral-phase high-performance liquid chromatography; NP-HPLC, normal-phase high-performance liquid chromatography; RP-HPLC, reverse-phase high-performance liquid chromatography; SMP, submitochondrial particles.

and walruses (*Pinnipeds*), (iv) sea otters (*Musteloidea*), and (v) polar bears (*Ursidae*). There are considerable differences between these species with respect to the level of dependence on the marine environment. Whales and dolphins strongly depend on an aquatic environment since they spend all stages of their lives in water and cannot survive on land. In contrast, seals, sea lions, and walruses feed in the water but breed and rest on land. Polar bears spend most of their time out of the water. In fact, they feed and reproduce on land or ice but looking for food, they occasionally enter the water. All marine mammals evolved from land-living animals millions of years ago [4]. For instance, whales and dolphins became aquatic some 50 million years ago [4] and comparative studies indicated that land-living even-toed ungulates and whales share a common ancestor [7]. In fact, among extant mammals hippopotamus is the closest relative for whales and dolphins [8]. Sea cows and pinnipeds (seals, sea lions, walruses) reentered the water 40 and 20 million years ago, respectively [4,9]. In contrast, sea otters are relative newcomers in marine life. About 2 million years ago, they evolved in the North Pacific and then spread down to the North American coast [4]. Although these animals reentered the marine environment only recently, they appear to be better adapted to aquatic life than seals, sea lions, and walruses, which must leave the water to give birth. Polar bears have diverged from a certain population of brown bears, which became isolated during pleistocenic glaciations [10,11]. Comparison of the mitochondrial DNA of polar bears and brown bears suggested that the two species diverged some 150 000 years ago and that some brown bear subspecies are more closely related to polar bears than to other brown bear subspecies. Despite this close evolutionary relation, polar bears are considered marine mammals but brown bears are not.

Eicosanoids and related compounds are lipid-signaling molecules, which have been implicated in cell physiology and in the pathogenesis of hyperproliferative, inflammatory, and neurodegenerative diseases [12–14]. They are biosynthesized from arachidonic acid and other polyenoic fatty acids (PUFAs) *via* three different pathways: (i) cyclooxygenase (COX) pathway leading to the formation of pro-inflammatory prostaglandins [15], (ii) lipoxygenase (ALOX) pathway leading to the formation of pro-inflammatory leukotrienes, but also to anti-inflammatory mediators such as resolvins and maresins [16], and (iii) cytochrome P450 (Cyt450) pathway leading to the formation of epoxyeicosanoids and vicinal fatty acid diols [17]. Although these biosynthetic pathways are well characterized in humans and other land-living mammals, little is known on eicosanoid biosynthesis in aquatic mammals. A PubMed search using the keywords 'marine mammals' and 'lipoxygenase' revealed some 70 hits but neither of these papers characterized the lipoxygenase pathway in marine mammals. In fact, to the best of our knowledge, it has never been explored systematically whether ALOX and COX isoforms do actually occur in aquatic mammals. To fill this gap, we first screened different publicly available sequence databases for ALOX- and COX-like sequences in marine mammals. Since we observed widespread occurrence of the two classes of enzymes in these animals, we selected species representing different subfamilies of marine mammals, for which complete ALOX15 sequences were available. These enzymes were expressed in pro- and eukaryotic overexpression systems and were characterized with respect to their catalytic properties. We found that all tested marine mammals express arachidonic acid 12-lipoxygenating ALOX15 orthologs and no 15-lipoxygenating enzymes could be identified. Thus, ALOX15 orthologs follow the Evolutionary Hypothesis of ALOX15 specificity. Since introduction of more space-filling amino acids at the triad positions altered the reaction specificity in favor of arachidonic acid 15-lipoxygenation, the enzymes follow the Triad concept.

Results

Classification of marine mammals and their evolutionary relatedness

Mammals (*Theria*) are classified into three major subfamilies (*Prototheria*, *Metatheria*, and *Eutheria*), and *Eutheria* are subdivided into *Xenarthra*, *Afrotheria*, *Euarchontoglires*, and *Laurasiatheria*. Sea cows are marine mammals, which are classified as *Afrotheria* and these animals are closely related to the African Elephant. In contrast, most extant marine mammals are classified as *Laurasiatheria*. These animals originated from the northern supercontinent *Laurasiatheria* involve several land-living species such as shrews, even-toed and odd-toed ungulates, pangolins, and large predators, but also marine mammals, such as whales and dolphins. Moreover, the large predators also involve a number of marine mammals such as seals, walruses, sea bears, and polar bears. In Fig. 1, a systematic representation of different laurasiaterian marine mammals and their evolutionary relation is given. This image also summarizes those laurasiaterian marine mammals, which were selected for more detailed structural and functional investigations.

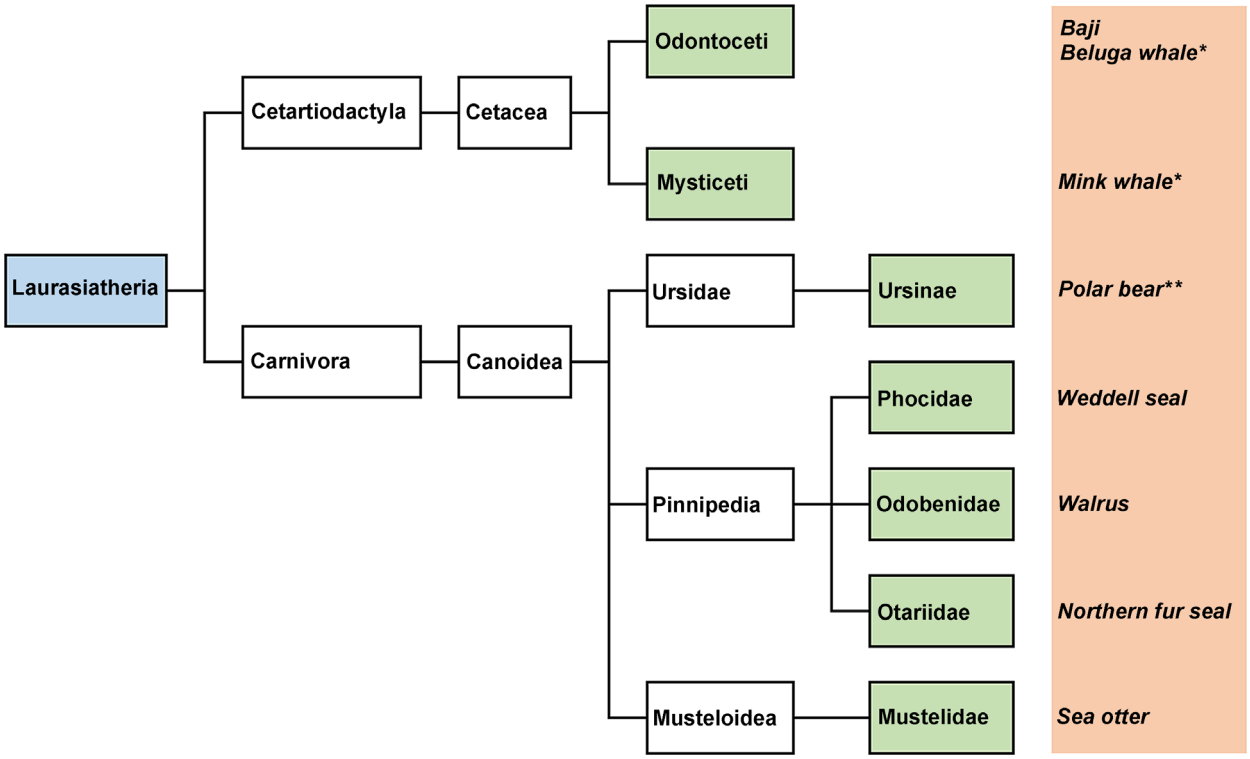

Fig. 1. Evolutionary relations of laurasiatherian marine mammals. Most marine mammals are classified as *Laurasiatheria*. *Cetacea* and *Canoidea* represent two major subcategories of marine mammals, and we selected the species given on orange background for functional studies. *For the species marked with an asterisk, we retrieved complete ALOX15 sequences but the enzymes are not expressed in pro- and eukaryotic expression systems. **The ALOX15 sequence of the polar bear is incomplete, and thus, this enzyme could neither be expressed. However, the available sequences for these enzymes allowed prediction of the reaction specificity based on the Triad concept.

Database searches and sequence alignments of eicosanoid-synthesizing enzymes

Although eicosanoids are known as physiologically relevant lipid mediators for more than 50 years, little is known about the biological relevance of these compounds in marine mammals. To explore the eicosanoid-synthesizing capacity of different marine mammals, we first screened the publicly available sequence databases (protein databases, genome databases) for the presence of cyclooxygenase (COX1, COX2) and lipoxygenase (ALOX15, ALOX15B, ALOX12, ALOX12B, ALOXE3, ALOX5) genes and judged the functionality of the corresponding enzymes identifying catalytically relevant amino acids.

Cyclooxygenases constitute key enzymes in the biosynthesis of prostaglandins, which have been implicated in a large number of physiological and pathophysiological processes [18–21]. The crystal structure of bovine COX1 has been solved [20], and dual amino acid sequence alignment with human COX1 identified catalytically important amino acids in the human ortholog (Table 1). Tyr384 is essential for the stereoselective hydrogen abstraction, which constitutes the rate-limiting step of the COX reaction. His206 (proximal) and His387 (distal) function as heme-binding ligands and, thus, are important for the peroxidase activity of this enzyme. COX1 is constitutively expressed in a large number of cells and tissues and is involved in the biosynthesis of physiological prostaglandins [18,21]. When we searched the public sequence databases (protein sequences and genomic sequences), we found that COX1 genes are present in all marine mammals tested (Table 1). Dual amino acid sequence alignments with human COX1 (Fig. S1a-n) indicated that the degree of amino acid identity of the identified COX1 orthologs with human COX1 varied between 50 and 90%. Moreover, in all sequences the catalytically important amino acids are conserved (Table 1) and these data suggested that the database sequences represent catalytically active enzymes.

COX2 is an inducible enzyme, which is expressed at high levels in stimulated inflammatory cells [18,19,21]. It is responsible for the biosynthesis of pro-inflammatory prostaglandins, and selective COX2 inhibitors have

Table 1. Presence of COX1 in different marine mammals. The amino acid sequences of the COX1 orthologs of different marine mammals were extracted from the protein database of the different mammals, and sequences were crosschecked using the corresponding genomic sequences. If required, corrections were introduced into the amino acid sequences based on the genomic data (*). In order to quantify the degree of amino acid conservation and to identify functionally relevant residues, dual amino acid sequence alignments were carried out with human COX1 (Fig. S1a-n). The deposited sequence of the polar bear and Weddell seal COX1 genes were incomplete (exons 1 + 2 are lacking), which might be related to a sequencing artifact.

Species	Accession number	Size[a]	Hydrogen abstraction	Heme-binding ligands
Human	NP_000953.2	599	Y384	H206 + H387
Sperm whale	XP_028349354.1	599*	Y384	H206 + H387
Narwhale	XP_029059492.1	601*	Y386	H208 + H389
Long-finned pilot whale	XP_030714606.1	599*	Y384	H206 + H387
Minke whale	XP_007178145.1	599*	Y384	H206 + H387
Killer whale	XP_004269299.1	599*	Y384	H206 + H387
Beluga whale	XP_022434509.1	599*	Y384	H206 + H387
Finless porpoise	XP_024618000.1	599*	Y384	H206 + H387
Baiji	XP_007468427.1	599*	Y384	H206 + H387
Pacific white-sided dolphin	XP_026960744.1	599*	Y384	H206 + H387
Weddell seal	XP_006739552.2	568	Y353	H175 + H356
Northern fur seal	XP_025743822.1	600*	Y385	H207 + H388
Walrus	XP_004411631.1	632	Y417	H239 + H420
Sea otter	XP_022356773.1	634	Y418	H240 + H421
Polar bear	XP_008697028.1	568*	Y353	H175 + H356

[a]Number of amino acids.

Table 2. Presence of COX2 in different marine mammals. The amino acid sequences of the COX2 orthologs of different marine mammals were extracted from the protein databases of the different mammals and from genome databases. Deposited protein sequences were crosschecked using the corresponding genomic sequences. *If required, minor corrections were introduced into the amino acid sequences based on the genomic data. In order to quantify the degree of amino acid conservation and to identify functionally relevant residues, dual amino acid sequence alignments were carried out with human COX2 (Fig. S2a-n).

Species	Accession number	Size[a]	Hydrogen abstraction	Heme-binding
Human	NP_000954.1	604	Y371	H193 + H374
Sperm whale	XP_007115359.2	604	Y371	H193 + H374
Narwhale	XP_029086928.1	604	Y371	H193 + H374
Long-finned pilot whale	XP_030728883.1	604	Y371	H193 + H374
Minke whale	XP_007167039.1	604	Y371	H193 + H374
Killer whale	XP_004275286.1	604	Y371	H193 + H374
Beluga whale	XP_022449092.1	604	Y371	H193 + H374
Finless porpoise	XP_024606955.1	604	Y371	H193 + H374
Baiji	XP_007451212.1	604	Y371	H193 + H374
Pacific white-sided dolphin	XP_026938795.1	604	Y371	H193 + H374
Weddell seal	XP_006730988.2	604*	Y371	H193 + H374
Northern fur seal	XP_025733679.1	604	Y371	H193 + H374
Walrus	XP_004405789.1	604	Y371	H193 + H374
Sea otter	XP_022364100.1	604	Y371	H193 + H374
Polar bear	XP_008695171.1	604	Y371	H193 + H374

[a]Number of amino acids.

been developed as potent anti-inflammatory drugs [22,23]. Functional COX2 genes were detected in all tested marine mammals, and the degree of amino acid identity with human COX2 varied between 70 and 90 % (Table 2). Dual amino acid sequence alignments with human COX2 (Fig. S2a-n) indicated that in the extracted sequences, functionally important amino acids are conserved and these data suggest functionality of these genes and the corresponding enzymes.

ALOX15 is one of the six human ALOX isoforms. It has been implicated in cell differentiation and maturation [24–27] but also in the pathogenesis of inflammatory, hyperproliferative, and neurological disorders [16,28,29]. Its crystal structure is known [30,31], the enzyme has well been characterized, and catalytically important amino acids have been identified [32,33]. Primates, which are ranked in evolution above gibbons (humans, chimpanzees, orangutans, gorilla), express 15-lipoxygenating ALOX15 orthologs. In contrast, mammals ranked lower than gibbons in evolution express 12-lipoxygenating enzymes and gibbons represent the evolutionary turning point [34,35]. When we searched the sequence databases, we detected ALOX15 orthologs in all marine mammals tested. The

degree of amino acid identity with human ALOX15 varied between 75% and 86 % (Table 3), and the triad determinants involved the Val(Ala)418 + Val(Ala)419 motif (Fig. S3a-n). These amino acid motifs are characteristic for arachidonic acid 12-lipoxygenating ALOX15 orthologs [35]. The Coffa determinant [33,36], which controls the stereochemistry of oxygen insertion, was an Ala in all cases (Fig. S3a-n), and thus, all enzymes were predicted to function as arachidonic acid 12S-lipoxygenating enzymes.

Human ALOX15B is an arachidonic acid 15-lipoxygenating enzyme [37] but its murine ortholog forms 8S-HETE as major arachidonic acid oxygenation product. As molecular reason for the altered reaction specificity of the two enzyme orthologs, a minor difference in the amino acid sequence has been identified [38]. Mutagenesis of the two amino acids located at positions 603 and 604 of mouse Alox15b (Jisaka determinants) to the corresponding amino acids present in human ALOX15B (His603Asp + His604Val) resulted in a 15S-lipoxygenating mutant, and the inverse mutagenesis strategy on human ALOX15B did also alter the reaction specificity [38]. The Triad concept is not applicable for mammalian ALOX15B orthologs [39]. Searching the sequence databases (protein and genomic sequences), we identified ALOX15B orthologs in all tested marine mammals (Table S1). Except for the polar bear, the iron ligands in ALOX15B are strongly conserved. Moreover, the Jisaka determinants were occupied by either the Asp + Val or the Asn + Ile motifs and the Coffa determinant was an Ala in all cases (Fig. S4a-n). This sequence information suggested that the ALOX15B orthologs of marine mammals might function as

Table 3. Presence of ALOX15 orthologs in different marine mammals. The amino acid sequences of ALOX15 orthologs of different marine mammals were extracted from the protein databases of the different mammals. These sequences were crosschecked by the corresponding genomic sequences, and if required, minor corrections were introduced into the amino acid sequences. In order to quantify the degree of amino acid conservation and to identify functionally relevant residues (iron ligands, triad determinants, stereo determinants), dual amino acid sequence alignments were carried out with human ALOX15 (Fig. S3a-n).

Species	Accession number	Size[a]	Iron ligands	Triad determinants	Coffa determinant
Human	NP_001131.3	662	H360 + H365 H540 + H544+I662	F353 + I418+ M419 + I593	A403
Sperm whale	XP_007109263.2	649	H379 + H384 H550 + 554+I672	F372 + A437+ A438 + I603	A423
Narwhale	XP_029066989.1	663	H362 + H367 Y541 + H545+I663	F353 + V418+ V419 + I593	A404
Long-finned pilot whale	XP_030718215.1	686	H362 + H367 H541 + H545+I663	F353 + V418+ V419 + I593	A404
Mink whale	XP_007166551.1	663	H362 + H367 H541 + H545+I663	F353 + V418+ V419 + I593	A404
Killer whale	XP_012387396.1	694	H394 + H399 H563 + H567+I685	F386 + V451+ V452 + I615	A437
Beluga whale	XP_022451845.1	663	H362 + H367 Y541 + H545+I663	F353 + V418+ V419 + I593	A404
Finless porpoise	XP_024592292.1	663	H362 + H367 Y541 + H545+I663	F353 + V418+ V419 + I593	A404
Baiji	XP_007457788.1	663	362 + H367 H541 + H545+I663	F353 + V418+ V419 + I593	A404
Pacific white-sided dolphin	XP_026986059.1	745	H362 + H367 H541 + H545+I663	F353 + V418+ V419 + I593	A404
Weddell seal	XP_030894829.1	663	H362 + H367 H541 + H545+I663	F353 + V418+ V419 + I593	A404
Northern fur seal	XP_025720066.1	663	H362 + H367 H541 + H545+I663	F353 + V418+ V419 + I593	A404
Walrus	XP_004398616.1	663	H362 + H367 H541 + H545+I663	F353 + V418+ V419 + I593	A404
Sea otter	XP_022380480.1	663	H362 + H367 H541 + H545+I663	F353 + V418+ V419 + I593	A404
Polar bear	XP_008686293.1	696	H394 + H399 H574 + H578+I696	F386 + V451+ V452 + I626	A437

[a]Number of amino acids.

arachidonic acid 15S-lipoxygenating enzymes. It should be stressed at this point that we did not express and functionally characterize any ALOX15B ortholog of marine mammals, and thus, this conclusion remains to be confirmed experimentally in subsequent expression studies.

Mammalian ALOX12 orthologs oxygenate arachidonic acid to 12S-HETE [40], and this reaction specificity is consistent with the predictions made on the basis of the Triad concept [Phe + Val(Ala)+Val (Ala)+Ile(Val)] (Table S2). Except for the polar bear, the iron ligands are well conserved but this might be related to the observation that the polar bear ALOX12 is apparently 150 amino acids bigger than that of other marine mammals. In mice, knockout studies have implicated this enzyme in platelet function [41] and in epidermal differentiation [42], but recent studies also suggested a role in hemostasis [43]. When we searched the sequence databases (protein and genomic sequences), we found ALOX12 orthologs in all marine mammals tested (Table S2). The degree of amino acid identity with human ALOX12 varied between 53% and 83%. The triad determinants involve the Val (Ala)+Val(Ala) motif, which is characteristic for arachidonic acid 12-lipoxygenating enzymes, and an Ala occupies the Coffa position (Fig. S5a-n). These data predict an arachidonic acid 12S-lipoxygenating enzyme for all tested marine mammals but experimental confirmation of this prediction is still pending.

ALOX12B and ALOXE3 are two ALOX isoforms, which are high level expressed in the skin, and thus, they are frequently referred to as epidermal ALOX isoforms. Human and murine ALOX12B orthologs are arachidonic acid 12R-lipoxygenating enzymes [44,45], which have been implicated in epidermal differentiation. Systemic knockout of the *Alox12b* gene induced malfunction of the epidermal water barrier, and newborns die within hours after birth because of dehydration [46]. Mutations in the human ALOX12B gene have been related to the pathogenesis of congenital ichthyosis [47], a hereditary skin disease characterized by excessive scaling. The Triad concept is not applicable to ALOX12B orthologs [39], but the enantioselectivity of these enzymes (12R-lipoxygenating) is consistent with the observation that a Gly occupies the Coffa position [48]. When we screened the protein databases of marine mammals for ALOX12B orthologs, we could not find such sequences in any of the tested *Cetaceaneae* (whales and dolphins). In a second step, we searched the genomic sequences of these animals for ALOX12B genes using the human ALOX12B gene as probe. Here again, we did not find ALOX12B genes (Table S3), and taken together, these data indicate that the genomes of whales and dolphins do not involve functional ALOX12B genes. *Cetaceans* do not bear furs, and the structure of their skin is different when compared to that of land-living mammals, such as mice and humans. On the other hand (Table S3), we did detect functional ALOX12B orthologs in fur-bearing marine mammals (Weddell seal, northern fur seal, walrus, sea otter, polar bear) and these enzymes exhibit a high (70–80%) degree of amino acid identity with human ALOX12B. All iron ligands are conserved, and a Gly occupies the Coffa position (Fig. S6a-e). These data suggest that these enzymes are catalytically active and that they might function as 12R-lipoxygenases. The lack of functional ALOX12B orthologs in *Cetaceans* might lead to the prediction that epidermal differentiation in these animals does not follow the mechanisms suggested for mice and humans [49,50].

ALOXE3 constitutes an epidermal ALOX isoform [51–53], which has been suggested to work in concert with ALOX12B during epidermal differentiation [49,50]. Systemic ALOXE3 deficiency also induces an ichthyosis-like phenotype in mice but the symptoms are less severe when compared to those observed for *Alox12b* knockouts [54]. Although recombinant ALOXE3 exhibits an arachidonic acid oxygenase activity (formation of racemic 5-HpETE, 7-HpETE, and 9-HpETE) in the presence of exogenous hydroperoxides and under hyperoxic conditions, it has been suggested to function *in vivo* as hydroperoxide isomerase [55–57]. When we explored the distribution of ALOXE3 orthologs in marine mammals (Table S4), we detected ALOXE3 genes in all tested species regardless of whether they are fur-bearing or not. After extraction of the cDNA sequences from the genomic data, we found that the degree of amino acid identity of these enzymes with human ALOXE3 varied between 70% and 75% and the iron ligands are conserved (Fig. S7a-n). As in human ALOXE3, an Ala occupies the Coffa position (Table S4), and taken together, these data suggest the functionality of these enzymes. However, our dual amino acid sequence alignments also indicated the presence of premature stop codons and/or frameshifting point mutations in the coding region of the ALOXE3 genes of whales and dolphins, which interrupt their open reading frames. Based on these sequence data, one can conclude that although *ALOXE3* genes are present in the genome of marine mammals, they do not encode for functional ALOXE3 orthologs in *Cetaceaneae*.

ALOX5 is the key enzyme in the biosynthesis of leukotrienes [58,59] and plays an important role in inflammatory diseases. The crystal structure of a

stabilized version of human ALOX5 has been solved [60], and knockout mice show a selective opposition to certain inflammatory insults [61]. When fed a lipid-rich diet, these animals develop aortic aneurisms [62] suggesting a role of this enzyme in vascular remodeling [63]. Searching the protein databases, we detected ALOX5 sequences in all marine mammals (Table S5). The degree of amino acid identity with human ALOX5 varied between 88% and 95%, and the iron ligands are well conserved (Fig. S8a-n). Moreover, the triad determinants were identical with those amino acids identified at these positions in human ALOX5 and these data suggest the functionality of the corresponding enzymes. However, for the sperm whale we identified a truncated version of ALOX5 (412 amino acids instead of 674 amino acids), which lacks internal parts of the enzyme. Since the lacking sequences involve functionally relevant amino acids, it is most probable that this truncated ALOX5 version is catalytically silent. However, it might well be that the apparent truncation is a result of a sequencing artifact.

Expression of selected ALOX15 orthologs of marine mammals

To test the functionality of ALOX15 orthologs of different marine mammals and to compare the corresponding enzymes with those of land-living mammals, we selected different species representing distinct evolutionary subclasses (Fig. 1). Unfortunately, no sequence information is currently available for sea cows and other afrotherian marine mammals. In contrast, for laurasiatherian marine mammals a number of complete ALOX15 sequences are available and we selected the following species for expression and functional characterization (Fig. 1): (i) baiji, beluga whale, and mink whale representing *Cetacea*; (ii) Weddell seal, walrus, and northern fur seal representing *Pinnipedia*; and (iii) sea otter representing *Musteloidea*. Unfortunately, no complete ALOX15 sequence data are currently available for the polar bear, and thus, no ALOX15 ortholog could be expressed. For expression, the coding regions of the cDNAs were cloned in the pET bacterial expression vector and recombinant ALOX15 orthologs were expressed as N-terminal hexa-his-tag fusion proteins. First, we tested by western blotting the presence of recombinant enzymes in the bacterial lysate supernatants using an antibody directed against the hexa-his-tag as probe. From Fig. 2A, it can be seen that the ALOX15 orthologs of baiji, (lane 1), northern fur seal (lane 4), walrus (lane 5), sea otter, and Weddell seal are present in variable amounts in the bacterial lysis supernatant. In contrast,

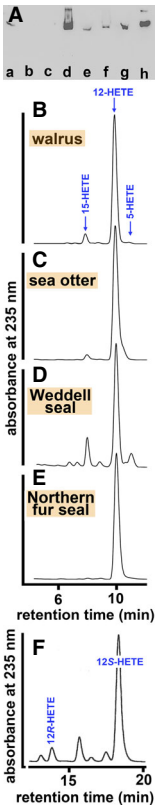

Fig. 2. Expression of ALOX15 orthologs of marine mammals in *E. coli*. The ALOX15 orthologs of selected marine mammals are expressed as recombinant his-tag fusion proteins in *E. coli* as described in Materials and Methods. A) Immunoblot analysis: 5 µL of the bacterial lysis supernatant was applied to SDS/PAGE; the separated proteins were blotted to a nitrocellulose membrane; and the blot was probed with an anti-his-tag antibody. To estimate the expression levels of the recombinant proteins, 250 ng of pure recombinant *M. fulvus* LOX [88] was applied for calibration purposes. Lane (a) baiji, lane (b) mink whale, lane (c) beluga whale, lane (d) northern fur seal, lane (e) walrus, lane (f) sea otter, lane (g) Weddell seal, lane (h) 250 ng of pure recombinant *M. fulvus* LOX. B-E) ALOX15 activity assays of different ALOX15 orthologs analyzed by RP-HPLC: Different volumes of the bacterial lysis supernatants were employed for the activity assays (see Materials and Methods) using arachidonic acid as substrate, and RP-HPLC analysis indicated the formation of specific ALOX15 products (12- and 15-HETE). The chromatograms were scaled for the highest product peak (12-HETE). F) Activity assay of baiji ALOX15 analyzed by NP-HPLC: Arachidonic acid oxygenation products formed by recombinant baiji ALOX15 were prepared by RP-HPLC and further analyzed by NP-HPLC (see Materials and Methods). In this experiment, a modified cell lysis protocol was employed (see Materials and Methods).

no immunoreactive material was detected in the lysis supernatants of beluga whale and mink whale.

Next, we performed activity assays using the bacterial lysate supernatants as enzyme source. For this purpose, aliquots of the lysate supernatant were

incubated with arachidonic acid for 8 min and the oxygenation products were analyzed by RP-HPLC. Here, we found the formation of specific lipoxygenase products by the ALOX15 orthologs of walrus (Fig. 2B), sea otter (Fig. 2C), Weddell seal (Fig. 2D), and the northern fur seal (Fig. 2E).

In these cases, the major oxygenation products comigrated in RP-HPLC with an authentic standard of 12-HETE. In addition, variable amounts of 15-HETE were also detected. No ALOX15 activity could be observed when the bacterial lysate supernatants of the beluga whale, the mink whale, and baiji were used as enzyme source. Together with the lacking western blot signals, these data suggest that the corresponding enzymes are either not well expressed in *E. coli* or that they have been deposited as denatured proteins in inclusion bodies. In fact, we observed large amounts of immunoreactive protein in the bacterial lysis pellets of mink whale and baiji. Although we performed a number of renaturation experiments, we could not restore the catalytic activity of these enzymes. No ALOX15 expression was observed for the ALOX15 ortholog of the beluga whale. To obtain functional data for baiji ALOX15, we employed a modified cell lysis protocol (see Materials and Methods). With this method, we obtained a catalytically active enzyme preparation and NP/CP-HPLC analysis of the oxygenation products revealed the formation of 12S-HETE as major oxygenation product (Fig. 2F). When we attempted to express the ALOX15 orthologs of the beluga whale and the mink whale in different eukaryotic overexpression systems (HEK293 cells, Sf9 insect cells), we never obtained sufficient amounts of catalytically active protein.

Finally, we tried to purify the recombinant enzymes by affinity chromatography on Ni-agarose. For some ALOX15 orthologs, this method worked very well and we obtained strongly enriched enzyme preparations. However, other ALOX15 orthologs could not be purified and we experienced a strong reduction of the catalytic activity. Thus, we decided not to purify the enzymes for more detailed catalytic characterization but use the lysate supernatants as enzyme source.

Functional characterization of recombinant ALOX15 orthologs

To compare the ALOX15 orthologs of marine mammals with human ALOX15, we quantified a number of enzyme characteristics, which have previously been determined for human ALOX15 [64]. Human ALOX15 is an arachidonic acid 15-lipoxygenating enzyme [65]. In contrast, the ALOX15 ortholog of the sea otter, which represents *Mustelidae* (Fig. 1), converts the same substrate mainly to 12-HETE (Fig. 2C). Small amounts of 15-HETE were also formed (Fig. 3A, right trace), and this dual reaction specificity is characteristic for mammalian ALOX15 orthologs [39,64,66,67]. NP-HPLC analysis (Fig. 3A, left trace) confirmed this finding, and chiral-phase HPLC indicated a strong preponderance of 12S-HETE over the corresponding R-enantiomer (Fig. 3A, middle trace).

Next, we determined the temperature dependence of arachidonic acid oxygenation by this enzyme and found a T_{opt} of 15 °C (Fig. 3B). At higher temperatures, the reaction rate declines suggesting a high degree of thermolability of the enzyme. From the data determined in the temperature range between 5 °C and 15 °C, we constructed the Arrhenius plot and calculated an activation energy of 32.3 kJ·mol^{-1} (Fig. 3C). The pH_{opt} of arachidonic acid oxygenation was 8.0 (Fig. 3D), and a K_M value of 26.9 μm was determined (Fig. 3E). Thus, the substrate affinity of the sea otter ALOX15 is comparable with that of the rabbit enzyme [68]. There was no significant increase in the reaction rate of arachidonic acid oxygenation (Fig. 3F) when the reaction was carried out under hyperoxic conditions, and these data suggest a high oxygen affinity of the enzyme.

Similar characterization studies were carried out for the ALOX15 orthologs of the northern fur seal (Fig. S9A–F) representing *Otariidae* (Fig. 1), Weddell seal (Fig. S10A–F) representing *Phocidae* (Fig. 1), and walrus (Fig. S11A-F) representing *Odobenidae* (Fig. 1). The kinetic parameters are summarized in Table 4. Unfortunately, there is no sufficient sequence information currently available in the databases to express and characterize the ALOX15 ortholog of the polar bear representing *Ursinae* (Fig. 1).

Membrane oxygenase activity of the ALOX15 orthologs of marine mammals

ALOX15 orthologs of different mammals exhibit biomembrane oxygenase activity [69–71]. To explore whether the ALOX15 orthologs of marine mammals are also capable of oxygenating membrane-bound polyenoic fatty acids, we incubated the recombinant enzyme preparations with mitochondrial membranes (submitochondrial particles, SMP) and analyzed by HPLC the oxygenation products in the hydrolyzed lipid extracts. From Fig. 4C, it can be seen that conjugated dienes are present in the hydrolyzed lipid extracts of SMPs treated with the ALOX15 ortholog of the northern fur seal. In no-enzyme (membranes only) controls, the conjugated diene peak was much

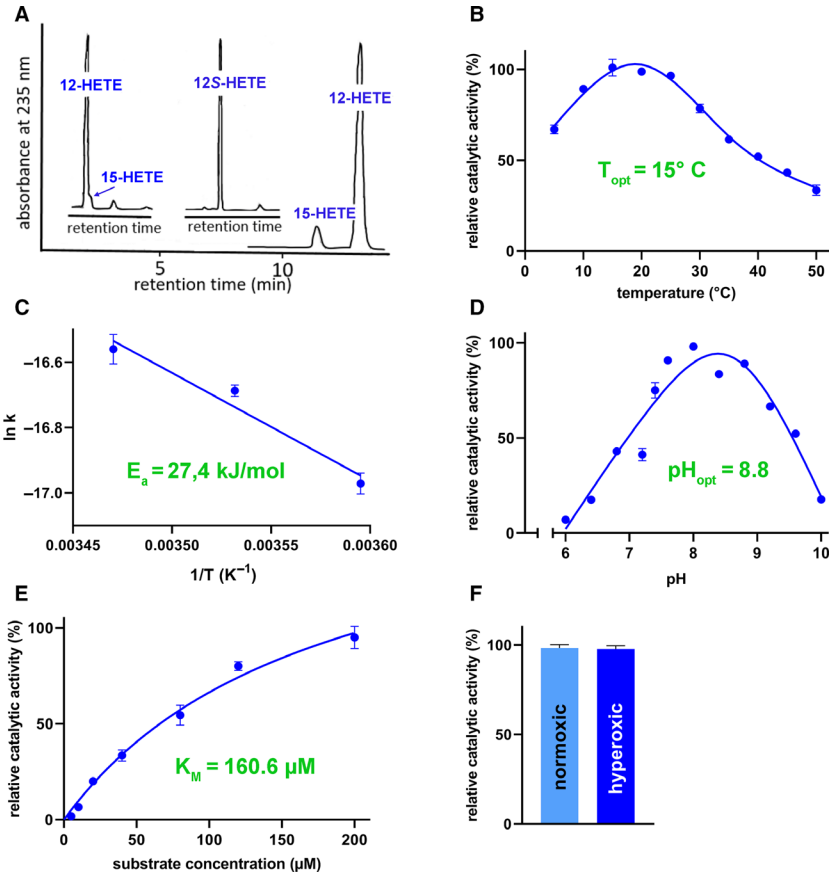

Fig. 3. Characterization of the catalytic properties of the ALOX15 ortholog of sea otter. The ALOX15 ortholog of sea otter was expressed as recombinant N-terminal hexa-his-tag fusion protein in *E. coli*, and aliquots of the bacterial lysis supernatant were used as enzyme source. All measurements were carried out at room temperature. (A) Reaction specificity: Activity assays were performed as described in Materials and Methods (pH 7.4, 100 µM arachidonic acid as substrate), and the reaction products were analyzed by RP-HPLC (right trace). The major conjugated dienes were fractionated and further analyzed by NP- (left trace) and CP-HPLC (middle trace). Taken together, these data indicate that 12S-HETE is the major oxygenation product of the ALOX15 ortholog of sea otter. (B) Temperature dependence: Arachidonic acid oxygenase activity assays were carried out at different temperatures and the formation of conjugated dienes during the incubation period was quantified as measure of the catalytic activity of the recombinant enzyme. The mean of the catalytic activities at 15°C was set 100%, and the relative activities measured at the other temperatures were quantified. At each temperature, 4 measurements were carried out and means were calculated. The standard deviations (SD) are represented by the error bars. (C) Arrhenius plots: The means of relative catalytic activities determined in the temperature ranging between 5° and 15°C (see panel B) were used to construct the Arrhenius plot and to determine the activation energy. (D) pH optimum: Activity assays were carried out at different pH and the mean of the catalytic activities measured at pH 8 was set 100%. To ensure homogeneity of the buffer mixtures at all pH, we mixed equal volumes of 10 mM phosphate buffer with 10 borate buffer and adjusted the corresponding pH at room temperature by the addition of 5 M HCl or 5 M KOH. At each pH value, 4 measurements were carried out and means were calculated. The standard deviations (SD) are represented by the error bars. (E) Substrate affinity studies: The arachidonic acid oxygenase activity was assayed at different substrate concentrations (room temperature, pH 7.4) and the K_m value was determined (SigmaPlot, Systat Software GmbH, Erkrath, Germany). At each substrate concentration, 4 measurements were carried out and means were calculated. The standard deviations (SD) are represented by the error bars. (F) Oxygen dependence of catalytic activity: Comparative oxygenase activity studies at two different oxygen concentrations were carried out. For normoxic measurements, the reaction buffer of the activity assays (room temperature, pH 7.4, 100 µM arachidonic acid) was saturated with air, which gives an approximate oxygen concentration of about 170 µm. For hyperoxic measurements, the reaction buffer was bubbled with pure oxygen gas for about 30 min, and then, the reaction was started by the addition of the enzyme preparation. At each condition, 4 measurements were carried out and means were calculated. The standard deviations (SD) are represented by the error bars.

lower (Fig. 4A) indicating the ALOX15 origin of the conjugated dienes. Following the chromatogram at 210 nm (Fig. 4B + D), we analyzed the polyenoic fatty acid content of the membranes and the chromatograms show that linoleic acid and arachidonic acid are the two major ALOX15 substrates present in

Table 4. Catalytic parameters of ALOX15 orthologs of different marine mammals using arachidonic acid as substrate. Kinetic studies were carried out as described in Materials and Methods, and the bacterial lysis supernatants were used as enzyme source

Species	Temperature optimum [°C]	activation energy [kJ·mol^{-1}]	pH optimum	K_M value [μM]
Sea otter	15	27.44	8	160.6
Weddell seal	15	32.33	7.6	62.9
Walrus	20	158.6	7.4	43.9
Northern fur seal	15	16.04	7.6	156.4

mitochondrial membranes. The chromatograms of the enzyme incubation (Fig. 4D) and the no-enzyme control (Fig. 4B) look very similar, and these data suggest that ALOX15 incubation does not oxygenate a large share of the polyenoic fatty acids. To quantify the degree of oxidation of the membrane lipids, we next determined the amounts of conjugated dienes (OH-PUFAs) and the amounts of polyenoic fatty acids (PUFAs) present in the hydrolyzed lipid extracts prepared from the incubations with (Fig. 4C + D) and without (Fig. 4A + B) ALOX15 treatment and calculated the OH-PUFA/PUFA ratios. Here, we found that 1.72% of the two major PUFAs were present as oxygenated derivatives after ALOX15 treatment. Similar values were obtained when these membranes were treated with native rabbit ALOX15 [69], recombinant human ALOX15 [64], or recombinant rat ALOX15 [67].

Identical experiments were carried out for the ALOX15 orthologs of Weddell seal, sea otter, and walrus, and the analytical data are summarized in Table 5. To further characterize the major oxygenation products, we pooled the products eluted in the HODE/HETE region of the four different ALOX15 orthologs, prepared the conjugated dienes formed, and analyzed them by combined normal-phase/chiral-phase (NP/CP-) HPLC. Here, we confirmed (Fig. 4E) that esterified 13-HODE and 12-HETE were the major conjugated dienes. Similar product patterns were identified when ALOX15 orthologs of other marine mammals were used as catalysts (Table 6).

Taken together, these data indicate that the ALOX15 orthologs of marine mammals exhibit membrane oxygenase activity and that esterified 13-HODE and 12-HETE are the major oxygenation products, when these enzymes oxygenate mitochondrial membrane lipids.

Mutagenesis studies on the triad determinants

Human ALOX15 is an arachidonic acid 15-lipoxygenating enzyme [72], whereas the mouse [73] and rat orthologs [67,74] are 12-lipoxygenating. The Triad concept explaining the variable reaction specificity of mammalian ALOX15 orthologs suggests that the side chain geometry of the triad determinants (Phe353, Ile418 + Met419, Ile593 of human ALOX15) is important for the reaction specificity. When small amino acids are located at the positions, which align with these amino acids of human ALOX15, the corresponding enzyme exhibits an arachidonic acid 12-lipoxygenating activity. In contrast, when more space-filling side chains are localized at these positions, the formation of 15-HETE is dominant [29]. To explore whether the ALOX15 orthologs of marine mammals follow the Triad concept, we first carried out an amino acid sequence alignments with human ALOX15 and identified the triad determinants (Fig. S3a-n). Here, we found that Phe353 (Borngraber determinant I) of human ALOX15 is strongly conserved in marine mammals. In contrast, at the Sloane positions (Ile418 + Met419 of human ALOX15) two small amino acids (Val or Ala) are located in marine mammals (Fig. S3a-n). These data suggest that the ALOX15 orthologs of marine mammals function as arachidonic acid 12-lipoxygenating enzymes and this conclusion is consistent with the results of our activity assays (Fig. 2). To test whether introduction of more space-filling residues at the triad determinants alters the reaction specificity of these enzymes in favor of arachidonic acid 15-lipoxygenation, we first expressed the following mutants of the northern fur seal ALOX15: Val418Ile, Val419Met, and Val418Ile + Val419Met. The analysis of the arachidonic acid oxygenation products is shown in Fig. 5.

The wild-type enzyme is a major 12-lipoxygenating enzyme (Fig. 2), and we confirmed this finding in our mutagenesis studies (Fig. 5). The Val418Ile mutant exhibits pronounced dual reaction specificity since similar amounts of 12- and 15-H(p)ETE are formed (Fig. 5). The Val419Met mutant prefers the formation of 12-H(p)ETE but compared with the wild-type enzyme, the share of 15-H(p)ETE formation was clearly augmented (Fig. 5). The Val418Ile + Val419Met double mutant functions as dominant 15-lipoxygenating enzyme, and the share of 12-H(p)ETE formation dropped down to about 10%.

The Triad concept also predicts that alterations in the reaction specificity induced by mutations of the Sloane determinants (Val418 + Val419) can be reversed, when the Borngraber I determinant (Phe353) is mutated [32]. To find out whether this is also the case for the ALOX15

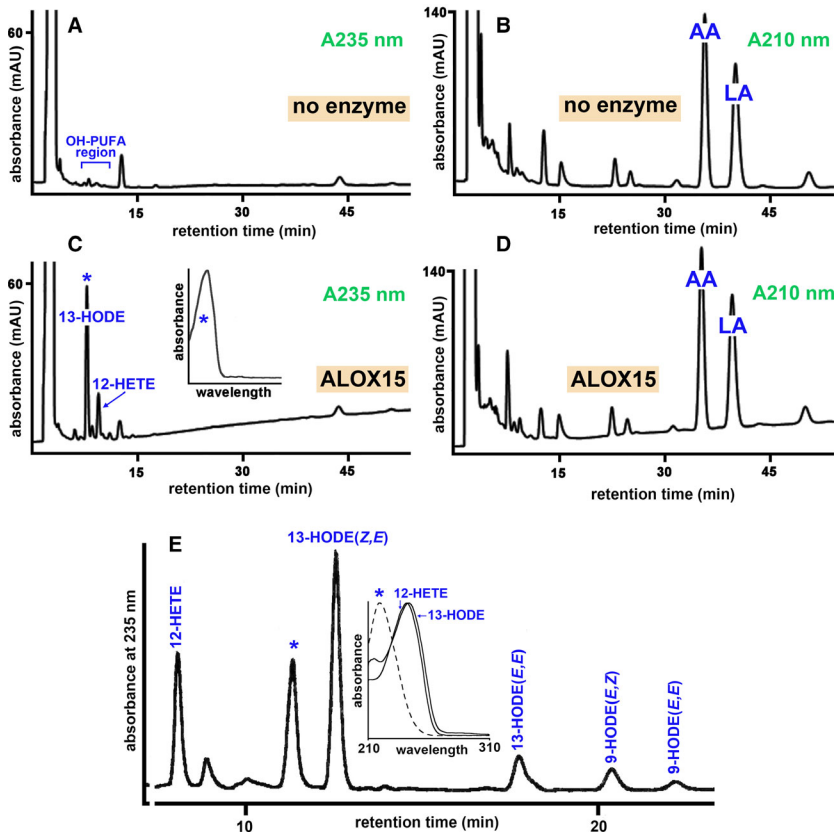

Fig. 4. Membrane oxygenase activity of the ALOX15 orthologs of marine mammals. Membrane oxygenase activity assays were carried out as described in Materials and Methods. The hydrolyzed lipid extracts were analyzed by RP-HPLC, and the absorbances at 235 nm (elution of conjugated dienes) and 210 nm (elution of PUFAs) were monitored. (A) No-enzyme control (membranes only), detection of conjugated dienes, (B) No-enzyme control (membranes only), detection of PUFAs. (C) ALOX15 of northern fur seal incubation, (D) ALOX15 of northern fur seal incubation, detection of PUFAs. (E) Normal-phase (NP-) HPLC analysis of the conjugated dienes formed during the membrane oxygenase activity assays carried out with the ALOX15 orthologs of Weddell seal, northern fur seal, sea otter, and walrus: Membrane oxygenase activity assays were performed with the ALOX15 orthologs of these marine mammals (see Materials and Methods). The conjugated dienes were prepared by RP-HPLC and pooled together. Solvent was evaporated; lipids were reconstituted in normal-phase HPLC solvent and analyzed by NP-HPLC (see Materials and Methods). Inset: The UV spectra of the products co-eluting with authentic standards of 12-HETE and 13-HODE. The peak marked with an asterisk (*) did not exhibit a typical diene chromophore, and control preparations indicated that it originated from the RP-HPLC solvent.

ortholog of the northern fur seal, we mutated in the Val418Ile + Val419Met double-mutant Phe353 to smaller amino acids (Leu, Ala). We found that the Phe353Leu + Val418Ile+Val419Met and the Phe353Ala + Val418Ile+Val419Met triple mutants catalyzed almost exclusive arachidonic acid 12-lipoxygenation (Fig. 5). Thus, introduction of less space-filling residues at Phe353 completely inverted the alterations in reaction specificity induced by Val418Ile + Val419Met exchange. Similar results were obtained when the ALOX15 orthologs of sea otter, walrus, and Weddell seal were taken through this experimental protocol (Table 7). Taken together, these data indicate that the Borngraber-1 (Phe353) determinant and the Sloane determinants (Val/Ala418 + Val/Ala419) functionally interact with each other and impact the reaction specificity of these enzymes in a concerted way. From these data, one can conclude that the Triad concept is applicable to ALOX15 orthologs of all marine mammals included in this study.

Discussion

Eicosanoid-synthesizing enzymes in marine mammals

Eicosanoids are lipid mediators that have been implicated in homeostasis but also in the pathogenesis of important diseases. COX-inhibitors are potent anti-inflammatory drugs [22], and leukotriene receptor

Table 5. Membrane oxygenase activity of ALOX15 orthologs of marine mammals. The membrane oxygenase activity assays were carried out as described in Materials and Methods, and the hydroxy PUFA/PUFA ratio in the hydrolyzed lipid extracts was quantified to judge the degree of oxygenation of the membrane ester lipids after ALOX15 treatment

Species	PUFA content (nmoles/sample)	OH-PUFA content (nmoles/sample)	OH-PUFA/ PUFA ratio (%)
Sea otter	193	1.2	0.6
Weddell seal	206	0.9	0.5
Walrus	208	1.4	0.7
Northern fur seal	261	4.5	1.7
No-enzyme control	209	0.2	0.1

Table 6. Product patterns formed when different marine mammal ALOX15 orthologs oxygenate the membrane lipids of mitochondrial membranes. The membrane oxygenase activity assays were carried out as described in Materials and Methods. The conjugated dienes formed (12-HETE, 13-HODE) were quantified by RP-HPLC as indicated in Fig. 4

Species	12-HETE (%)	13-HODE (%)
Sea otter	34.1	65.9
Weddell seal	28.5	71.5
Walrus	38.7	61.3
Northern fur seal	33.3	66.7

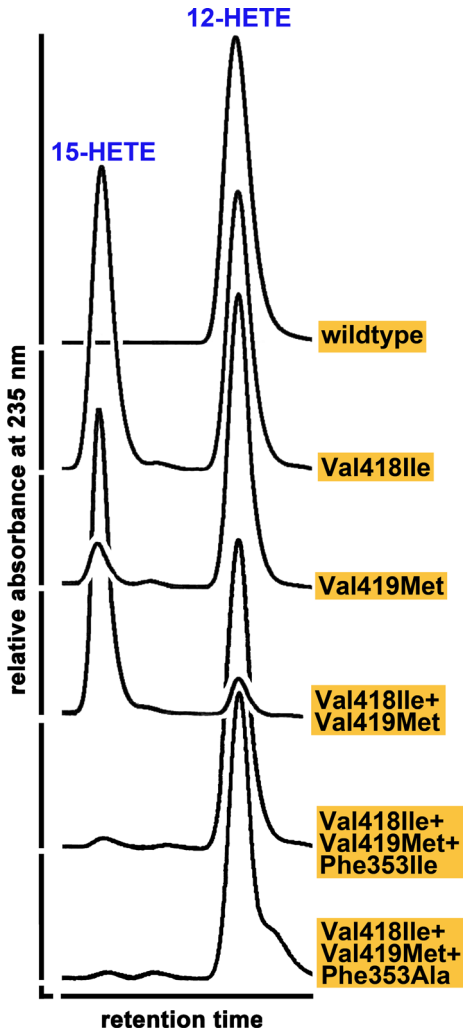

Fig. 5. Mutagenesis of the triad determinants of the ALOX15 ortholog of northern fur seal. Enzyme variants were prepared as described in Materials and Methods, and activity assays were carried out. Aliquots of the activity assays were analyzed by RP-HPLC, and the chromatograms were scaled to the height of the dominant conjugated dienes (12- or 15-HETE). Partial chromatograms of the OH-PUFA region are shown.

antagonists [75] are available for prescription as anti-asthmatics. Despite our comprehensive knowledge on eicosanoid biology in mice and humans, little is known on the role of these mediators in marine mammals. In this study, we systematically searched the publicly available protein databases for eicosanoid-synthesizing enzymes in marine mammals and found that COX1, which is responsible for the biosynthesis of physiological prostaglandins, is present in all tested marine mammals (Table 1). According to the results of our sequence alignments, the enzymes detected should be functional since the functionally important amino acids are well conserved. COX2 orthologs have also been detected in all marine mammals tested (Table 2). Here again, the functional amino acids are strongly conserved suggesting catalytic activity of the corresponding enzymes.

ALOX15 orthologs have been detected in all marine mammals tested (Table 3), and these data indicate a high degree of distribution of this enzyme in these animals. As for COX2, catalytically important amino acids (iron ligands, triad determinants, stereo determinants) are well conserved, and thus, the enzymes should be catalytically active. This conclusion is supported by the catalytic activity of the recombinant ALOX15 enzymes expressed in our study. Other ALOX isoforms (ALOX15B, ALOX12, ALOX12b, ALOXE3, ALOX5) were also detected in most marine mammals (Table S1–S5), and here again, the catalytically important amino acids are conserved. Interestingly (Table S3), we did not find *ALOX12B* genes in any of the nine *Cetacean* species

Table 7. Applicability of the Triad concept for ALOX15 orthologs of marine mammals. ALOX15 orthologs of marine mammals were mutated, and the single, double, and triple mutants were employed for activity assays (see Materials and Methods). The major oxygenation products were identified by RP-HPLC strategy. The 12S-HETE/15S-HETE ratios are given in the table. Values < 1 indicate 12-HETE as major oxygenation product; values < 1 indicate 15-HETE as major oxygenation product. n.d., not determined; these mutants could not be expressed as catalytically active proteins

Species	Wild-type	Val418Ile	Val419Met	Val418Ile+ Val419Met	Val418Ile+ Val419Met+ Phe353Leu	Val418Ile+ Val419Met+ Phe353Ala
Sea otter	7.60	0.66	1.50	0.04	0.78	3.10
Weddell seal	78.60	1.00	2.80	0.11	99.0	10.6
Walrus	54.30	2.31	3.29	0.13	n.d.	n.d.
Northern fur seal	99	1.06	8.53	0.15	99	99

(whales and dolphins) tested and this conclusion was based on the data obtained screening the protein databases and genomic sequences of these animals. *Cetaceae* do not carry a fur, and the skin of these animals is differently structured when compared to fur-bearing mammals. Interestingly, ALOX12B orthologs were present in all tested fur-bearing marine mammals (Table S3) and these data suggest that this enzyme may be related to fur development. ALOX12B and ALOXE3 have previously been implicated in epidermal differentiation [47,49,50] in mice and humans. In fact, Alox12b knockout mice die from dehydration shortly after birth [76], and thus, the enzyme has been implicated in the formation of the epidermal barrier preventing water evaporation. Since *Cetaceaneae* are born in the water and spend their entire life in aqueous surroundings, they do not need an epidermal water evaporation barrier. Thus, there might be no evolutionary pressure on the ALOX12B genes in *Cetaceaneae*, and the lack of such pressure might have led to the disappearance of these genes. Interestingly, ALOXE3 genes are present in the genomes of the tested *Cetaceaneae* (Table S4). This finding was rather surprising, since ALOX12B and ALOXE3 orthologs have been implicated in the formation of the epidermal water barrier. However, when we explored the genomic ALOXE3 sequences of *Cetaceaneae* in more detail we detected premature stop codons and/or frameshifting point mutations in the coding regions of the corresponding genes (Fig. S7) and these data suggest that these genes do not encode for functional proteins. Thus, according to these sequence data *Cetaceaneae* may not express functional ALOXE3 orthologs.

Comparison of ALOX15 orthologs of marine mammals with those of land-living mammals

In general, ALOX15 orthologs exhibit a broad substrate specificity accepting most naturally occurring polyenoic fatty acids as substrate. The ALOX15 orthologs of marine mammals oxygenate arachidonic acid to 12S-HETE (Fig. 2), and 13S-HODE was the major product of linoleic acid oxygenation (data not shown). When the two fatty acids were simultaneously supplied as constituents of biomembranes, 13S-HODE formation was dominant (Fig. 4).

The K_M value for arachidonic acid oxygenation varied between 44 and 160 μm for the different marine mammalian ALOX15 orthologs tested. These data values are higher than the K_M values determined for native rabbit and recombinant human ALOX15 for linoleic acid [64,68]. It should, however, been stressed that we assayed the fatty acid oxygenase activity in the absence of any detergent, which lowers the water solubility of the substrate fatty acids and, thus, its availability for the enzymes. In a similar experimental setup (no detergent), we determined similar K_M values for arachidonic acid for the ALOX15 orthologs of *P. aeruginosa* [71] and *T. belangeri* [77].

Compared with the ALOX15 orthologs of land-living mammals, the thermostability of the enzymes of marine mammals is rather low. At room temperature, the enzymes undergo rapid inactivation and even on ice the enzyme preparations loose catalytic activity. This catalytic instability might also be discussed as reason why the enzymes inactivate during affinity chromatography purification. The molecular basis for the catalytic instability has not been explored.

Mammalian ALOX and COX isoforms exhibit a high affinity for atmospheric oxygen, and the oxygen K_M varied between 10 and 26 μm [78]. In contrast [79], oxygen affinity of the 15-lipoxygenating *P. aeruginosa* ALOX was much lower (K_M of 0.4 mM). When we studied the impact of higher oxygen concentrations on the rate of arachidonic acid oxygenation, we did not observe increased rates under hyperoxic conditions (Fig. 3, Fig. S9, S10, S11). Although we did not exactly quantify the oxygen K_M, the lacking increase

in catalytic activity under hyperoxia suggests that under normoxic conditions, the enzymes are oxygen saturated. Thus, these data suggest a high oxygen affinity of the ALOX15 orthologs of marine mammals.

All tested ALOX15 orthologs of marine mammals are capable of oxygenating membrane ester lipids (Fig. 4, Table 5). This catalytic activity was first described for rabbit ALOX15 [80] and has later been confirmed for the enzyme orthologs of other land-living mammals [64,67,70]. Unfortunately, for ALOX15 orthologs of marine mammals no information on this catalytic property has been available. Here, we showed that the tested ALOX15 orthologs of marine mammals exhibit a membrane oxygenase activity but compared with the fatty acid oxygenase activity this catalytic activity was 10-fold less efficient. This finding is consistent with the observations made for other mammalian ALOX15 orthologs [81]. Although the mechanistic basis for this phenomenon has not been explored, it may be possible that the membrane proteins protect the membrane lipids from the ALOX15 attack.

The ALOX15 orthologs of marine mammals follow the Triad concept

The Triad concept of ALOX15 specificity suggests that three clusters of amino acids (triad determinants) are important for the reaction specificity [32]. In human and rabbit ALOX15, these determinants are Phe353 (Borngraber-1 determinant), Ile418 + Met419 (Sloane determinant), and Ile593 (Borngraber-2 determinant) [32,82,83]. In all marine mammalian ALOX15 orthologs, a Phe aligns with Phe353 of human ALOX15 (Fig. S3a-n). Ile418 + Met419 of human ALOX15 aligns with Val + Val, Val + Ala, or Ala + Val motifs in marine mammalian ALOX15 orthologs. Thus, small amino acids are present at the Sloane determinants. Ile593 of human ALOX15 is conserved in all marine mammalian ALOX15 orthologs. Taken together, these sequence data predict arachidonic acid 12-lipoxygenating ALOX15 orthologs for all marine mammals. To explore whether the triad determinants functionally interact with each other, we introduced the more bulky amino acids present in human ALOX15 at these positions into the marine enzyme orthologs and observed a gradual change in the reaction specificity favoring arachidonic acid 15-lipoxygenation (Table 7). In fact, the Val418Ile + Val419Met double mutants were dominantly 15-lipoxygenating. When we introduce smaller amino acids at the Borngraber-1 position (Val418Ile + Val419Met+ Phe353Leu, Val418Ile + Val419Met+Phe353Ala), the changes induced by mutagenesis of the Sloane determinants (Val418Ile + Val419Met) were reversed and dominantly 12-lipoxygenating triple mutants resulted (Fig. 5). Taken together, these data indicate that the Triad concept explains the reaction specificity of the ALOX15 orthologs of marine mammals and that the triad determinants functionally interact with each other.

The reaction specificity of the ALOX15 orthologs of marine mammals supports the Evolutionary Hypothesis of ALOX15 specificity

The Evolutionary Hypothesis of ALOX15 specificity suggests that mammals ranked in evolution above gibbons (humans, chimpanzee, gorillas, orangutans) express an arachidonic acid 15-lipoxygenating ALOX15. In contrast, lower primates and other mammals express arachidonic acid 12-lipoxygenating enzymes [35,84]. There are rare exceptions, but more than 90 % of all functionally characterized mammalian ALOX15 orthologs follow this concept [35,84]. If this hypothesis is applicable for marine mammals, their ALOX15 orthologs should function as arachidonic acid 12-lipoxygenating enzymes. Analysis of the sequence data we extracted from the databases (Table 3) indicates that the Sloane determinants are occupied by small amino acids (Val/Ala418, Val/Ala419), and on the basis of the Triad concept [35], one can predict arachidonic acid 12-lipoxygenating activity for the 14 marine mammal ALOX15 orthologs we found in the database. Four of them have been expressed as recombinant enzyme and functional characterization confirmed this prediction (Fig. 3). Thus, the reaction specificities of the functionally tested ALOX15 orthologs of marine mammals support the Evolutionary Hypothesis.

MATERIALS AND METHODS

Chemicals

The chemicals were purchased from the following vendors: acetic acid from Carl Roth GmbH (Karlsruhe, Germany); sodium borohydride from Life Technologies, Inc. (Eggenstein, Germany); antibiotics and isopropyl-β-thiogalactopyranoside (IPTG) from Carl Roth GmbH (Karlsruhe, Germany); restriction enzymes from Thermo Fisher Scientific-Fermentas (Schwerte, Germany); the *E. coli* strain Rosetta2 DE3 pLysS from Novagen (Merck-Millipore, Darmstadt, Germany); and *E.* coli strain XL-1 from Stratagene (La Jolla, CA, USA). Oligonucleotide synthesis was performed at BioTez Berlin Buch GmbH (Berlin, Germany). Nucleic acid sequencing was carried out at Eurofins Genomics Germany GmbH (Ebersberg, Germany). HPLC-grade methanol, acetonitrile, and water were purchased

from Fisher Scientific. Authentic HPLC standards of HETE isomers (15S/R)-HETE, 15S-HETE, 12(S/R)-HETE, 12S-HETE, 5S-HETE) and the polyenoic fatty acids used as ALOX substrates were purchased from Cayman Chem. [distributed by Biomol (Hamburg, Germany)].

In silico database searches and sequence alignments

Initially, we extracted the amino acid sequences of ALOX and PGHS orthologs of marine mammals from the public protein database (https://www.ncbi.nlm.nih.gov/protein) using the key words 'lipoxygenase' and 'prostaglandin H synthase'. In a second step, we searched the genomic sequences deposited in the genome databases of the different marine mammals using the genomic sequence of human PGHS and ALOX genes. The genomes of all the marine mammals explored in this study have been sequenced completely by next-generation sequencing techniques, and the different sequence reads were properly assembled. The genome coverage of the assemblies ranges from to 27-fold to 248-fold and, thus, were of high quality. The data are accessible to the public and can be downloaded from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/assembly/). The BLAST 2.10.0 + standalone software package was used for searching ALOX- and PGHS-like sequences in the genomes and to process the downloaded data. Initially, the makeblastdb program that converts fasta files to BLAST database files was used for creating a separate database for each species. Species-specific ALOX- and PGHS-like sequences were identified by screening each organism database using the corresponding sequences of the orthologous human genes employing the tblastn/blastn programs. The sequence hits identified were then analyzed on DNASTAR Lasergene (DNAS Inc., Madison, WI, USA), and the complete PGHS and ALOX sequences for each organism were identified. From the genomic sequences, we extracted the amino acid sequences and the protein database sequences were corrected for the genomic sequences if differences were observed. For amino acid alignments, we used the online tool https://www.ebi.ac.uk/Tools/psa/emboss_needle/.

Cloning of ALOX15 orthologs of marine mammals

To test the functionality of selected ALOX15 orthologs of marine mammals, we first extracted the cDNAs from the genomic sequence and optimized the coding regions for prokaryotic expression. Single amino acids missing in the extracted coding sequences were replaced by comparison with the sequences of the next relatives (mink whale, baiji, beluga). A *Sal*I restriction site was introduced immediately upstream of the starting ATG of the his-tag fusion construct, and a *Hind*III recognition sequence was designed downstream of the stop codon. Internal *Hind*III and *Sal*I sites were eliminated by silent nucleotide exchanges, and the construct was chemically synthesized (BioCat GmbH, Heidelberg, Germany). For prokaryotic expression, the construct was excised from the synthesizing vector (pUC57) and cloned into the expression plasmid pET28b (Novagen/Merck, Darmstadt, Germany). Recombinant expression plasmids were tested for the ALOX15 inserts by *Sal*I + *Hind*III digestion, and the final expression constructs were sequenced (Eurofins Genomics Germany GmbH, Ebersberg, Germany).

Bacterial expression of ALOX15 orthologs

Competent bacteria (Rosetta 2 DE3 pLysS) were transformed with 100ng of recombinant expression plasmids, and the cells were grown overnight on kanamycin/chloramphenicol containing agar plates. An isolated growing clone was selected and two 1 mL bacterial liquid cultures (LB medium with 50 $\mu g \cdot mL^{-1}$ kanamycin/35 $\mu g \cdot mL^{-1}$ chloramphenicol) were grown at 37°C. This preculture was added to a 50 mL main culture, and the cells were grown to reach an optical density (A_{600}) of 0.10–0.15. The culture medium [glucose-free MSM with added trace elements] [85] was supplemented with 40 $g \cdot L^{-1}$ dextrin, 0.24 $g \cdot L^{-1}$ tryptone/peptone, and 0.48 $g \cdot L^{-1}$ yeast extract. Antibiotics as well as 100 µL 1:20 diluted antifoam 204 (Sigma, Deisenhofen, Germany) and 50 µL Glucoamylase from Aspergillus niger (Amylase AG 300L, Novozymes, Bagsværd, Denmark) were added, and then, the main cultures were started in Ultra Yield flasks (Thomson Instrument Company, Oceanside, USA). After the OD_{600} had reached values above 5, expression of the recombinant his-tag fusion proteins was induced by the addition of 1 mM (final concentration) IPTG, 60 mg tryptone/peptone, 120 mg yeast extract, and 75–100 µL Glucoamylase were added. Then, the cultures were maintained at 22 °C for 18 h at 230–250 rpm agitation. Bacteria were harvested, the resulting pellet was reconstituted in a total volume of 5 mL PBS, and bacteria were lysed by sonication (digital sonifier, W-250D Microtip Model 102, 50% maximal sonication amplitude; Branson Ultraschall, Fürth, Germany). Debris was spun down (15 min, 15 000 ***g***, 4 °C), and the lysate supernatants were employed as enzyme source. For more drastic bacterial lysis (baiji ALOX15), high-pressure homogenization (Avestin Emulsiflex C5, Ottawa, Canada) was used.

Eukaryotic expression of ALOX15 orthologs in Sf9 cells

The expression was performed as described [84]. Briefly, the coding sequence was ligated into the pFastBac HT vector and the bacmid as well as the recombinant baculovirus

generated according to the manufacturer's instructions (Bac-to-Bac® Baculovirus Expression System, Invitrogen Life Technologies/Thermo Fisher, Schwerte, Germany). Protein expression was initiated in Sf9 cells (ATCC® CRL-1711) cultured using Insect XPRESS Medium (Biozym, Hessisch Oldendorf, Germany) supplemented with 4mM glutamine and 0.5% FBS. The cells were infected and subsequently incubated at 27°C and 120–130 rpm on a shaking platform. After 72 h (30% dead cells), the cells were harvested by centrifugation and lysed by sonication and the lysate supernatant after centrifugation was used as enzyme source.

SDS/PAGE and western blot

SDS/PAGE was performed as described in Ref. [77]. In brief, a 7.5% polyacrylamide gel was run with ProSieve Ex running buffer (Lonza Group Ltd., Basel, Switzerland) in a Bio-Rad electrophoretic chamber, and afterward, the proteins were transferred on a Protran BA 85 Membrane (Carl Roth GmbH, Karlsruhe, Germany). The membrane was blocked, washed, and finally incubated with an anti-His-HRP antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) for 1–2 h at room temperature. After washing steps, the membrane was developed with SERVA-Light Polaris CL HRP WB Substrate Kit for 5 min at room temperature. Chemiluminescence was detected on a FUJIFILM Luminescent Image Analyzer LAS-1000plus & Intelligent Dark Box II. For each sample, 5 μL of the lysate supernatant was applied and denatured using ProSieve ProTrack Dual Color Protein Loading Buffer + DTT for 3 min at 95 °C.

Site-directed mutagenesis

About 10–50 ng of plasmid DNA was incubated with the specific primer pair (1 μL of 5 μm solution each) and 12.5 μL of Pfu Ultra II Hot Start 2 × PCR Master Mix (Agilent Technologies, Waldbronn, Germany) in a total volume of 25 μL adjusted with sterile water. The PCR protocol was designed as follows: 95 °C for 1-min initial denaturation, cycle: 95 °C for 30s > 55 °C for 60s> 68 °C for 10 min; altogether, 18 cycles were applied. Subsequently, the parent DNA was digested with 1 μL DpnI (Thermo Scientific, Schwerte, Germany) for 30min, followed by denaturation at 80 °C for 10 min. About 8 μL of the PCR sample was used for transformation of 100 μL of *E. coli* XL-1 Blue competent cells (Agilent Technologies Inc., Santa Clara, USA). After incubation for 30 min on ice, the cells were heat-shocked for 45 s at 42 °C and kept on ice for 2 min, and then, 400 μL of SOC medium was added. After 1-h incubation at 37 °C and 180 rpm, the cells were plated on an LB-agar plate supplemented with 50 $\mu g \cdot mL^{-1}$ kanamycin (for pET28b) or 100 $\mu g \cdot mL^{-1}$ ampicillin (for pFastBac HT) and incubated overnight at 37 °C. Four clones were selected for liquid culture in 2 mL of LB medium, and plasmid DNA was prepared using the NucleoSpin Plasmid Kit (Macherey & Nagel, Düren, Germany). One clone was selected for sequencing (Eurofins Genomics Germany GmbH, Ebersberg, Germany).

Activity assays and HPLC analysis

To assay the catalytic activity of the recombinant ALOX15 orthologs, variable amounts of the bacterial lysis supernatants were added to 0.5 mL of PBS containing 100 μm arachidonic acid as substrate. After 8 min of incubation, the hydroperoxy fatty acids were reduced by the addition of 1 mg of solid sodium borohydride, and after 5 min, the reaction mixture was acidified with 45 μL of concentrated acetic acid. Then, the proteins were denatured by the addition of 0.5 mL acetonitrile and precipitates were removed by centrifugation. Aliquots (50–300 μL) of the clear supernatants were injected to RP-HPLC analyses on a Shimadzu instrument, which was connected to a Hewlett Packard diode array detector 1040 A. Metabolites were separated on a Nucleodur C18 Gravity column (Macherey-Nagel, Düren, Germany; 250 × 4 mm, 5 μm particle size) coupled with a corresponding guard column (8 × 4 mm, 5 μm particle size). A solvent system consisting of acetonitrile:water: acetic acid (70 : 30 : 0.1, by vol) was used at a flow rate of 1 $mL \cdot min^{-1}$.

For more detailed analysis, the conjugated dienes were prepared by RP-HPLC and further analyzed by normal-phase HPLC (NP-HPLC) and/or chiral-phase HPLC (CP-HPLC). Normal-phase HPLC was performed on a Nucleosil 100-5 column (250 × 4.6 mm, 5 μm particle size) using the solvent system n-hexane/2-propanol/acetic acid (100/2/0.1, by volume) at a flow rate of 1 $mL \cdot min^{-1}$. 12-HETE enantiomers were resolved on a Chiralpak AD-H column (Daicel Corp., Osaka, Japan) with a solvent system consisting of n-hexane/methanol/ethanol/acetic acid (96 : 3 : 1 : 0.1, by vol, 1 $mL \cdot min^{-1}$).

Membrane oxygenase activity

To test the membrane oxygenase activity of the ALOX15 orthologs, we incubated different volumes of the bacterial lysis supernatants in 0.5 mL PBS with submitochondrial particles (1.4 $mg \cdot mL^{-1}$ final membrane protein concentration). After 15 min, the reaction was stopped by the addition of 1 mg of sodium borohydride. Then, the sample was acidified with acetic acid, total lipids were extracted [86], and the ester lipids were hydrolyzed. After acidification, aliquots of the hydrolysates were analyzed by RP-HPLC and the chromatograms were followed simultaneously at 235 nm (conjugated dienes) and at 210 nm (nonoxidized polyenoic fatty acids). Quantifying the peak areas of the

major polyenoic fatty acids (LA + AA) and the conjugated dienes formed, we calculated the hydroxy PUFA/PUFA ratio, which constitutes a suitable measure for the degree of oxygenation of the membrane ester lipids [69].

Statistics

Statistical calculations and figure design were performed using MS Excel 2016 or GRAPHPAD PRISM version 8.00 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

FR, HK, and DH designed the study and planned the experiments. FR, KRK and DH performed the database searches, and SS and DH designed the expression strategies and the expression vectors. FR and SS prepared the enzymes and characterized the enzyme preparations. HK drafted the manuscript, and all co-authors contributed to the preparation of the submitted version.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Presence of ALOX15B orthologs in different marine mammals.
Table S2. Presence of ALOX12 orthologs in different marine mammals.
Table S3. Presence of ALOX12B orthologs in different marine mammals.
Table S4. Presence of ALOXE3 orthologs in different marine mammals.
Table S5. Presence of ALOX5 orthologs in different marine mammals.
Figure S1. Amino acid alignments of COX1 orthologs of different marine mammals with human COX1.
Figure S2. Amino acid alignments of COX2 orthologs of different marine mammals with human COX2.
Figure S3. Amino acid alignments of ALOX15 orthologs of different marine mammals.
Figure S4. Amino acid alignments of ALOX15B orthologs of different marine mammals.
Figure S5. Amino acid alignments of ALOX12 orthologs of different marine mammals.
Figure S6. Amino acid alignments of ALOX12B orthologs of different marine mammals.
Figure S7. Amino acid alignments of ALOXE3 orthologs of different marine mammals.
Figure S8. Amino acid alignments of ALOX5 orthologs of different marine mammals.
Figure S9. Characterization of basic catalytic properties of the northern fur seal ALOX15.
Figure S10. Characterization of basic catalytic properties of the Weddell seal ALOX15.
Figure S11. Characterization of basic catalytic properties of the walrus ALOX15.