Lipoxygenase pathways in *Homo neanderthalensis*: functional comparison with *Homo sapiens* isoforms

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Abstract  Lipoxygenases (LOX) have been implicated in biosynthesis of pro- and anti-inflammatory mediators, and a previous report suggested compromised leukotriene signaling in *H. neanderthalensis*. To search for corresponding differences in leukotriene biosynthesis, we screened the Neandertal genome for LOX genes and found that, as modern humans, this archaic hominin contains six LOX genes (nALOX15, nALOX12, nALOX5, nALOX15B, nALOX12B, and nALOX3) and one pseudogene. In the Neandertal genome, 60–75% of the amino acids of the different human LOX isoforms have been identified, and the degree of identity varies between 96 and 99%. Most functional amino acids (iron ligands, specificity determinants, calcium and ATP-binding sites, membrane-binding determinants, and phosphorylation sites) are well conserved in the Neandertal LOX isoforms, and expression of selected neandertalized human LOX mutants revealed no major functional defects. However, in nALOX12 and nALOX3, two premature stop codons were found, leading to inactive enzyme species. These data suggest that ALOX15, ALOX5, ALOX15B, and ALOX12B should have been present as functional enzymes in *H. neanderthalensis* and that in contrast to lower nonhuman primates (*M. mulatta*) and other mammals (mice, rats), this ancient hominin expressed a 15-lipoxygenating ALOX15. Expression of ALOX3 and ALOX12 was compromised, which might have caused problems in epidermal differentiation.

Lipoxygenases (LOX) constitute a heterogeneous family of lipid-peroxidizing enzymes that catalyze dioxygenation of free and/or esterified polyunsaturated fatty acids to their corresponding hydroperoxy derivatives (1–3). In mammals they have been implicated in cell development and maturation, but they also play a role in inflammatory and hyperproliferative diseases (1). LOX sequences have been identified in bacteria (4, 5) and eukarya (1, 6, 7), but they are apparently absent in archaea (2). Originally, mammalian LOXs have been categorized with respect to their positional specificity of arachidonic acid oxygenation, but recently sequence-based classification systems have been introduced (1, 2). In humans, six functional LOX genes (ALOX15, ALOX15B, ALOX12, ALOX12B, ALOX5, and ALOX3) exist that encode for six LOX isoforms (1). In contrast, there are seven complete *Alox* genes in mice, as the *Alox12* gene, which is an inactive pseudogene in humans, represents a functional gene in mice (1, 2). Knockout experiments of the *Alox12* gene as well as epidemiological studies in naturally occurring *Alox12B* mutants (8, 9) implicated this LOX isoform in epidermal differentiation. In contrast, *Alox15* (10), *Alox12* (11), and *Alox5* (12) knockout mice do not show major phenotypic defects unless challenged in special ways.

*H. neanderthalensis* is the closest evolutionary relative of today’s human beings, and in ancient times, they were spread out over large parts of Europe and western Asia (13). The earliest Neandertal fossils were assigned to a developmental period 400,000 years ago (14). The youngest Neandertal fossils have been dated to some 30,000 years ago (15), but there is evidence that these Pleistocene hominids may have survived in southwestern Europe (Gibraltar) until about 25,000 years ago (16). During the later parts of their history, Neandertals settled as far east as southern Siberia and as far south as the Middle East (14). During their existence, Neandertal individuals presumably got in contact with modern humans, and the discussion of whether Neandertals interbred with anatomically modern humans has been controversial. There are morphological and genetic evidences supporting and opposing this concept (13, 15). Unfortunately, the sequence of the mitochondrial Neandertal genome, which has been sequenced for several individuals (17, 18), consistently falls outside the variation found in today’s human beings. Thus, it does not provide...
conclusive evidence for interbreeding. However, the recently published draft sequence of the nuclear genome suggests that Neandertals are likely to have had a role in the genetic ancestry of today’s humans outside of Africa (19). Moreover, comparison of the genomic sequences of *H. sapiens* and *H. neanderthalensis* indicated a number of genomic regions that are different between ancient and modern humans (19). Among them was the cysteinyl leukotriene receptor 2 (CLTR2), which was mutated in the Neandertal genome. Although no direct functional studies have been carried out, the sequence data suggest that Neandertals might have suffered from compromised leukotriene signaling (19).

To judge the capability of Neandertal individuals to produce eicosanoids, we searched the Neandertal genome for LOX sequences and found that the six functional LOX genes identified in *H. sapiens* are conserved in the Neandertal genome. When we aligned the amino acid sequences of these genes with the corresponding human orthologs (hALOX), we found that corresponding isoforms share a high degree of amino acid identity (96–99%). Most amino acids that have previously been reported to be of functional importance for hALOX isoforms are conserved in nALOX isoenzymes. However, in nALOX12 and nALOXE3, we identified two premature stop codons, which interrupt the open reading frame.

### MATERIALS AND METHODS

**Chemicals**

The chemicals used were obtained from the following sources: arachidonic acid (5Z,8Z,11Z,14Z-eicosatetraenoic acid) from Serva (Heidelberg, Germany); HPLC standards of 5(±)-HETE, 8(±)-HETE, 9(±)-HETE, 11(±)-HETE, 12(±)-HETE, and 15(±)-HETE from Cayman Chemical (distributed by Biomol, Hamburg, Germany); sodium borohydride, ampicillin from Life Technologies, (Heidelberg, Germany); HPLC standards of 5(±)-HETE, 8(±)-HETE from Carl Roth GmbH (Karlsruhe, Germany). HPLC solvents were from Baker (Deventer, the Netherlands). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). 5(±)-HETE and 8(±)-HETE were from Carl Roth GmbH (Karlsruhe, Germany); sodium borohydride, ampicillin from Life Technologies, Inc. (Eggenstein, Germany); chloramphenicol from Sigma-Aldrich, St. Gallen (Switzerland); and isopropyl-b-thiogalactopyranoside (IPTG) from Carl Roth GmbH (Karlsruhe, Germany). HPLC solvents were from Baker (Deventer, the Netherlands). Restriction enzymes were purchased from New England Biolabs (Schwalbach, Germany). Oligonucleotide synthesis was performed at BioTez (Berlin, Germany), and nucleic acid sequencing was carried out at Eurofins MWG Operon (Ebersberg, Germany). The *E. coli* strain XL-1 blue was purchased from Stratagene (La Jolla, CA), and the *E. coli* strain BL21 (DE3) pLysS was from Invitrogen (Carlsbad, CA).

**Sequence extraction and alignments**

The *H. neanderthalensis* DNA was extracted largely from three Neandertal bones, each about 40,000 years old, from the Vindija Cave in Croatia: Vi33.16, Vi33.25, and Vi33.26 and their sequence are accessible on the UCSC Genome Browser (http://genome.ucsc.edu/). This portal provides access to sequence data and alignments with the reference human genome. Our alignments were carried out with the Genomatix software DiAlign (http://www.genomatix.de).

**Bacterial expression of hALOX isoenzymes**

To express human LOX isoforms as his-tag fusion proteins, the coding regions of the corresponding cDNAs were amplified by PCR, and the PCR fragments were cloned into different pro-caryotic expression vectors. Because of technical reasons, the N-terminus of the recombinant enzyme, including the hexameric his-tag sequence, was elongated by additional amino acids. A C-terminal restriction site (*HindIII*) was introduced immediately after the ATT stop codon for the recombinant enzyme species. Details on the expression vectors and on the cloning strategies of hALO15, hALOX12, hALOX5, and hALOX15B are given in the corresponding original publications (20–22).

**Site-directed mutagenesis**

Site-directed mutagenesis was performed using the Quick-ChangeTM Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, the Netherlands). Competent bacteria were transformed with the mutated expression plasmids, the cells were plated on selection agar plates, one of the growing bacterial clones was isolated, and the LOX insert was sequenced. Next, competent bacteria were retransformed with the mutant plasmid, and a well-separated colony was selected for enzyme preparation.

**Activity assays**

LOX activity in the stroma-free bacterial lysis supernatants was assayed by RP-HPLC quantification of arachidonic acid oxygenation products. Aliquots of the bacterial lysis supernatant were incubated for 10 min at room temperature with 0.1 mM arachidonic acid in 0.5 ml of PBS. The hydroperoxy compounds formed were reduced with sodium borohydride to the corresponding hydroxy derivatives, the mixture was acidified to pH 3, and 0.5 ml of ice-cold methanol was added. The protein precipitate was spun down, and aliquots of the clear supernatant were injected to RP-HPLC.

**HPLC analytics**

HPLC of the LOX products was performed on a Shimadzu instrument recording the absorbance at 235 nm. Reverse phase-HPLC was carried out on a Nucleodur C18 Gravity column (Marchery-Nagel, Düren, Germany; 250 × 4 mm, 5 µm particle size) coupled with a guard column (8 × 4 mm, 5 µm particle size). A solvent system of methanol/water/acetic acid (85/15/0.05, by volume) was used at a flow rate of 1 ml/min.

**Immunoblotting**

To normalize the LOX content in the lysis supernatant, aliquots of the supernatants were mixed with SDS sample buffer and loaded on SDS-PAGE. After electrophoresis, the separated proteins were electroblotted to a nitrocellulose membrane, and the blot was visualized using an anti-his tag antibody fused with horseradish peroxidase (Invitrogen, Carlsbad, CA). The intensity of the immunoreactive band at 75 kDa was quantified densitometrically, and expression of the wild-type enzyme was set 100%.

### RESULTS

**Classification of observed amino acid differences**

The University of California Santa Cruz (UCSC) Genome Browser Database contains genomic sequences for three independent Neandertal fossil specimens; the overall genome coverage coefficient was 1.3 (19). However, the combined individual sequences were incomplete when compared with the *H. sapiens* genome. Overlapping arrangement of all available nALOX coding sequences and amino acid alignments with the corresponding human enzymes indicated that the degree of coverage varied between 60% and 74% for the different nALOX isoenzymes (Table 1). For those amino acids, which were specified in
both genomes, the degree of sequence identity varied between 96% and 99% for the different ALOX isoforms (Table 1), but even for the most similar enzyme (nALOX15), we found a number of amino acid exchanges that might have impacted the functionality of the enzymes. For each LOX isoform, these amino acid differences were categorized according to their degree of reliability in three different groups (Table 1): i) Confirmed differences (+++, blue background in the figures; these amino acid differences were found in two of the three sequenced individual Neandertal fossil specimens); ii) unconfirmed differences (++, green background in the figures; these differences were observed in only one fossil specimen; for the other fossil specimens, there were no sequence data for the corresponding genomic region); and iii) uncertain differences (+, yellow background). In addition, we found two further amino acid differences (G111N and P384F), which were localized at the beginning or at the end of a sequence fragment; therefore, the likelihood of a sequencing artifact appears to be higher).

**ALOX15 (12/15-LOX)**

Amino acid alignment of hALOX15 and nALOX15 indicated that from the 662 amino acids identified for hALOX15, 489 (74%) were identified in the Neandertal genome. Assuming that the number of amino acid residues in hALOX15 and nALOX15 is identical, 173 amino acids (26%) could not be identified in nALOX15 (Table 1). About 30% of the unidentified amino acids are localized in the N-terminal β-barrel domain. These differences may not be of major functional relevance, as N-terminus truncation of hALOX15 does not lead to major functional alterations (22). The 489 amino acids, which were identified in the Neandertal genome, share a high degree of identity with the corresponding residues of hALOX15. Only 5 amino acids were different, indicating a degree of identity of 99%. Among the amino acid differences (Fig. 1), one exchange (T375I) showed up in the sequences of two different fossils specimens (Vi33.16 and Vi33.26), and thus, it was considered a confirmed difference (+++, blue background). Two additional differences (G25 and R640W) showed up in the sequences obtained from only one fossil (+, green background). In addition, we found two further amino acid differences (G111N and P384F), which were localized at the beginning or the end of sequenced DNA fragments. According to our nomenclature, these differences were classified as uncertain (+, yellow background).

After this global comparison, we searched the nALOX15 sequence for amino acid residues, which have previously been shown to be of functional relevance for this LOX isoform. All LOXs identified so far contain a redox active metal ion [iron (1, 2, 3) or manganese (23)] as a prosthetic group. Rabbit ALOX15 (rALOX15) contains a nonheme iron, which is ligated by 4 His and the N-terminal Ile (24). As indicated in Fig. 1, all protein iron ligands are conserved in nALOX15, suggesting a similar iron ligand sphere as in rabbit ALOX15.

Human ALOX15 exhibits a dual reaction specificity, as it converts arachidonic acid to a mixture of 15S- and 12S-HETE in a ratio of about 10:1 (25, 26). In contrast, the mouse ortholog (27) produces an inverse product pattern (15-HETE/12-HETE ratio of 1:9). The structural basis for the variable reaction specificities of ALOX15 isoforms has been explored (28), and a triad of sequence determinants was identified (2, 29). The triad concept suggests that the side-chain geometry of three critical amino acids (F352, I417/M418, and I592 of rALOX15) is decisive for the positional specificity of this enzyme. If space-filling amino acids are located at these positions, the enzyme introduces dioxygen at C15 of the arachidonic acid backbone. In contrast, if fewer space-filling residues are present, dominant 12-lipoxygenation results. From Fig. 1, it can be seen that the triad determinants identified for hALOX15 are strictly conserved in nALOX15; therefore, it can be concluded that nALOX15 constitutes a 15-lipoxygenating enzyme.

**TABLE 1. Properties of nALOX isoforms and comparison with hALOX enzymes**

| LOX Gene | Trivial Name of Protein | Length hALOX | Chromosome Localization | Amino Acids<sup>a</sup> Specified (%) | Amino Acid<sup>b</sup> Identity (%) | Identified Differences<sup>c</sup> | Stop Codons |
|----------|-------------------------|--------------|-------------------------|-------------------------------------|-----------------------------------|-------------------------------|-------------|
| nALOX15  | 12/15-LOX               | 662          | 17                      | 74                                  | 99                                | +++                           | 0           |
| nALOX15B | 15-LOX2 isof orm d      | 676          | 17                      | 65                                  | 99                                | ++                           | 2           |
| nALOX12  | p124LOX                 | 663          | 17                      | 63                                  | 96                                | +                            | 3           |
| nALOX3   | 5-LOX isof orm 1        | 674          | 10                      | 60                                  | 99                                | +                             | 0           |
| nALOX12B | 12R-LOX                 | 701          | 17                      | 75                                  | 98                                | +                             | 0           |
| nALOX3 (T1) | eLOX3 isof orm 2      | 711          | 17                      | 75                                  | 97                                | +                             | 4           |
| nALOX3 (T2)<sup>d</sup> | eLOX3 isof orm 1      | 843          | 17                      | 71                                  | 97                                | +                             | 5           |

The cDNA sequences of the different LOX isoforms were extracted from the *H. neanderthalensis* genome database, and the amino acid sequences were deduced. Amino acid sequence alignments with human LOX isoforms are shown in Figs. 1–6.

<sup>a</sup>Among the amino acids specified for *H. neanderthalensis*, more than 96–99% are identical with those of *H. sapiens*.

<sup>b</sup>Amino acids that are different between *H. neanderthalensis* and *H. sapiens* (amino acid exchanges) were categorized into three groups: +++ indicates confirmed exchanges (these amino acid exchanges are indicated by overlapping sequences of two different fossil specimens originating from two different *H. neanderthalensis* individuals); ++ indicates unconfirmed exchanges (these amino acid exchanges were concluded from the sequence of a single fossil specimen; no sequence data for this position is available from any of the other fossils explored); and + indicates uncertain mutations (these amino acid exchanges were concluded from the sequence of a single fossil specimen and the corresponding amino acid was located at the beginning or at the end of a sequenced DNA fragment).

<sup>c</sup>For hALOX3 and nALOX3, additional longer transcripts have been identified. Translation of these transcripts may be initiated at methionine residues (bold) localized upstream the "normal" translational initiation site (see Fig. 6B).
Multiple sequence alignments of a large number of LOX isoforms suggested that most S-LOXs carry an Ala at a critical position, whereas R-LOXs contain a Gly instead (30). Mutagenesis studies on a large number of different S-LOXs indicated that an Ala-to-Gly exchange increases the share of R-HETE isomers (3). The molecular basis for the observed specificity alterations is not entirely clear, but it was hypothesized that the larger Ala side chains may block oxygen penetration to the proR site of the fatty acid reaction intermediate. Although this concept (Coffa concept) is not strictly applicable to all LOX isoforms (31, 32), it has predictive value. Unfortunately, the corresponding amino acid (A403 in hALOX15) was not identified for nALOX15 (Fig. 1), and thus, the enantioselectivity of this LOX isoform cannot be specified.

R402 of hLOX15 has been suggested to interact with the carboxylic group of the fatty acid to arrest this part of the substrate at the active site (33) and R402L exchange has been suggested to lead to impairment of substrate binding as indicated by a drop of $K_M$. However, this residue is not conserved in other LOX isoforms; therefore, it may not be of general functional relevance. In nALOX15, this amino acid was not specified (Fig. 1).

LOXs are capable of directly interacting with biomembranes (1). Rabbit (34) and human ALOX15 (35) employ membrane-bound phospholipids as substrates. For rALOX15 membrane binding, determinants have been suggested in both the N-terminal and the C-terminal domains, and mutagenesis of these residues impaired membrane binding (36). When we compared rALOX15 with hLOX15, we found that Y15, L70, and L71 (N-terminal domain), and K180 and L194 (C-terminal domain) of hALOX15 aligned with the membrane-binding determinants of rALOX15. The residues aligning with L70 and L71 of hALOX15 were not identified in nALOX15, but Y15, K180, and L194 were all conserved (Fig. 1).

To explore the possible functional consequences of selected amino acid exchanges, we partly neandertalized hALOX15 by introducing selected single amino acid exchanges identified during sequence alignment. As indicated in Table 2, two of the neandertalizing single mutations (G2S and T375I) exhibited an increased catalytic efficiency.

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Unfortunately, we experienced problems in expressing the R640W mutant; therefore, we cannot comment on its activity.

ALOX15B (15-LOX2)

The human LOX isoform ALOX15B was discovered in 1997 (37). There are alternatively spliced transcripts, and we initially employed variant d for sequence comparison (Fig. 2A), which encodes for the longest enzyme isoform. Amino acid alignment indicated that from the 676 (isoform d) amino acids of hALOX15B, 440 (65%) were identified in the Neandertal genome. Of the unidentified amino acids, 34% are localized in the N-terminal β-barrel domain. The 440 amino acids, which were identified in the Neandertal genome, share a high degree of identity with the corresponding residues of hALOX15B. Here we found six amino acid differences, indicating a sequence identity of 99%. Among the different amino acids (Fig. 2), there was no confirmed (+++) exchange, but we identified four unconfirmed (+++, green background) amino acid differences (D20G, V167M, A225T, and D352N). Amino acid alignment of hALOX12 indicated that from the 663 amino acid residues in hALOX12 and nALOX12 (Table 1). About 40% of the unidentifiable amino acids are localized in the N-terminal β-barrel domain. As indicated in Fig. 3, the majority of the amino acids of the N-terminal β-barrel domain were not specified for nALOX12. Nevertheless, the 419 amino acids, which were identified in nALOX12, share a high degree of identity with the corresponding residues of hALOX12. Eighteen amino acids were different (Fig. 3), indicating an amino acid sequence identity of about 96%. Two of these amino acid differences (P254L and Q261R) are conserved in nALOX15B (Fig. 2). Unfortunately, in the corresponding Neandertal transcript (nALOX15B-d), this amino acid was not specified (Fig. 2B); therefore, the enantioselectivity of the corresponding enzyme cannot be predicted.

ALOX12 (pl12-LOX)

ALOX12 was the first animal lipoxygenase discovered almost 40 years ago (40). Amino acid alignment of hALOX12 and nALOX12 (Fig. 3) indicated that from the 663 amino acids identified for hALOX12, 419 (63%) were identified in the Neandertal genome. Assuming that the number of amino acid residues in hALOX12 and nALOX12 is identical, 244 amino acids (37%) were not identified in nALOX12 (Table 1). About 40% of the unidentified amino acids are localized in the N-terminal β-barrel domain. As indicated in Fig. 3, the majority of the amino acids of the N-terminal β-barrel domain were not specified for nALOX12. Nevertheless, the 419 amino acids, which were identified in nALOX12, share a high degree of identity with the corresponding residues of hALOX12. Eighteen amino acids were different (Fig. 3), indicating an amino acid sequence identity of about 96%. Two of these amino acid differences (P254L and Q261R) were found in two fossil specimens (Vi33.25 and Vi33.26); therefore, they were considered confirmed difference (+++, blue background). Ten additional amino acid exchanges (G111S, A113T, N234K, N363S, Q511Stop, S523N, Q524Stop, T537I, T560I, and A622V) showed up in the sequences of single fossil specimens; and thus, they were unconfirmed (+++, green background).

### Table 2. Enzymatic properties of neandertalized hALOX single mutants

| LOX Isoform | Mutant    | Related Specific Activity (%) | Major Product (%) |
|-------------|-----------|-------------------------------|------------------|
| ALOX15      | Wild-type | 100                           | 15-HETE (86)     |
|             | G2S       | 114                           | 15-HETE (82)     |
|             | T375I     | 217                           | 15-HETE (88)     |
|             | R640W     |                                | Not expressed    |
| ALOX12      | Wild-type | 100                           | 12-HETE (>99)    |
|             | G111S     | 64                            | 12-HETE (>99)    |
|             | A113T     | 57                            | 12-HETE (>99)    |
|             | P254L     | 41                            | 12-HETE (>99)    |
|             | Q261R     | 58                            | 12-HETE (>99)    |
|             | N363S     | 172                           | 12-HETE (>99)    |
|             | Q511Stop  | <1                            | ND               |
|             | A622V     | 170                           | 12-HETE (>99)    |

Neandertal single mutants of human LOX isoforms were expressed in E. coli (5 ml liquid culture). Enzymatic activity was determined by quantifying the reaction products by RP-HPLC and LOX protein content was estimated by immunoblotting. Catalytic activity of the wild-type enzyme was set 100%. ND, no products detected (inactive mutant).
A

| ALOX15B-a | MAEFPVRVSTQAPGFLTGW5VSIVGTR | GESIFFPLDQLWKEPTAGAECFPYTLR | 60 |
| ALOX15B-b | NAERVKXXXGAQQAGSTGVWVSVIVOX | XXXXXXXXXXXXETAGAAXXX | 120 |
| ALOX15B-c | VGYRLLVHAPFVPLPLPFLPOAWCFR | WPQITTPVGDELFPCYWLEQSTLY | 180 |
| ALOX15B-d | QGKVYKXQXXXXXGQX | WKNQPGWPPSEKDEYDAELNEKX | 240 |
| ALOX15B-e | NNRVFDGAQSNMGGKDLRIGKLR | NNNQPGWPPSEKDEYDAELNEKX | 300 |

**Fig. 2.** Dual amino acid alignment of hALOX15B and nALOX15B. The amino acid sequences of the two LOX isoforms were aligned with the DiAlign software. The amino acids, which have not been specified in the Neandertal genome, are indicated by X on gray background. Color coding is explained in the Fig. 1 legend. In addition, brown background indicates positional specificity determinants (39).

In hALOX12, the nonheme iron is liganded by three His, one Asn, and the C-terminal Ile (Fig. 3). Except for the C-terminal Ile, all protein iron ligands are conserved in nALOX12, suggesting a similar iron ligand sphere as present in the human enzyme.

Human ALOX12 exhibits a singular positional specificity, as it converts arachidonic acid almost exclusively to 12-HETE (40, 41), and the triad concept can partly be applied for this enzyme (38). From Fig. 3, it can be seen that three of the four triad determinants are strictly conserved between hALOX12 and nALOX12. Only the residue aligning with P352 of hALOX12 is not specified in nALOX12. Similarly, the enantioselectivity determinant (30) is not specified in nALOX12; therefore, no prediction can be made on enantioselectivity of this enzyme.

R402 of hALOX15 has been suggested to interact with the carboxylic group of the fatty acid substrate when it binds at the active site (33). A similar role might be suggested for the corresponding Arg of hALOX12. As indicated in Fig. 3, Arg402 of hALOX12 is conserved in nALOX12.

To explore the possible functional consequences of selected amino acid exchanges, we partly neandertalized hALOX12 by introducing single amino acid exchanges identified during sequence alignment. As indicated in Table 2, most of the neandertalizing single mutations did not dramatically alter the rate of arachidonic acid oxygenation. In fact, the two confirmed amino acid differences (P254L and Q261R) both exhibited about 50% residual catalytic activity, and similar values were obtained for other differences. N363S and A622V exchange led to an even more active enzyme species (Table 2). As expected, introduction of the premature stop codon at Q511 completely abolished the catalytic activity.

**ALOX5 (5-LOX)**

ALOX5 activity was first described in human polymorphonuclear leukocytes (42), and its genomic sequence was published in 1989 (43). Amino acid alignment of hALOX5 and nALOX5 (Fig. 4) indicated that from the 674 amino acids of hALOX5, 402 (60%) were identified in the Neandertal genome. Assuming that the number of amino acid residues in hALOX5 and nALOX5 is identical, then background). Two of these nucleotide exchanges introduced premature stop codons, which interrupt the open reading frame. Furthermore, we found six additional amino acid exchanges (M123I, F228V, P456S, W468Stop, R500Q, and P648L) that were localized at the beginning or the end of sequenced DNA fragments. According to our nomenclature, these differences were classified as uncertain (+, yellow background).

In hALOX15, the nonheme iron is liganded by three His, one Asn, and the C-terminal Ile (Fig. 3). Except for the C-terminal Ile, all protein iron ligands are conserved in nALOX15, suggesting a similar iron ligand sphere as present in the human enzyme.

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To explore the possible functional consequences of selected amino acid exchanges, we partly neandertalized hALOX15 by introducing single amino acid exchanges identified during sequence alignment. As indicated in Table 2, most of the neandertalizing single mutations did not dramatically alter the rate of arachidonic acid oxygenation. In fact, the two confirmed amino acid differences (P254L and Q261R) both exhibited about 50% residual catalytic activity, and similar values were obtained for other differences. N363S and A622V exchange led to an even more active enzyme species (Table 2). As expected, introduction of the premature stop codon at Q511 completely abolished the catalytic activity.

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/H9252-barrel domain of hALOX5, calcium-binding residues (N44, D45, and E47) and membrane-inserting amino acids (W13, W75, and W102) have been identified; site-directed mutagenesis of these residues impaired membrane binding (47). Unfortunately, none of these residues was specified in nALOX5, so no prediction can be made on its membrane-binding capacity.

The substrate-binding pocket of hALOX5 is an elongated cavity (48, 49), which appears to be sealed at both ends. The bulky side-chains of F178 and Y182, which are not present at this position in rALOX15, close the cavity at one end (FY-cork). The opposite opening may be blocked by the side chains of W148, and thus, substrate penetration appears to be sterically hindered. Two mechanisms have been suggested by which arachidonic acid can enter the active site:

i) movement of the FY-cork (decorking)

ii) rotation of the Trp147 side chain. These amino acids are well conserved in nALOX5 (Fig. 4); and therefore, similar mechanisms of substrate binding may be predicted for this enzyme.

When human leukocytes are activated, ALOX5 is phosphorylated by the activity of different protein kinases; three potential phosphorylation sites (S272, S524, and S664) have been identified (50). Phosphorylation of S664 is catalyzed by ERK2 and apparently activates the enzyme (51). Phosphorylation of S272 inhibits nuclear export of hALOX5 (52), and in contrast, phosphorylation of S524 inhibits nuclear import (53). S272 of hALOX5 was conserved in nALOX5, but the other potential phosphorylation sites were not specified.

272 amino acids (40%) were not identified in nALOX5 (Table 1). About 27% of the unidentified amino acids are localized in the N-terminal β-barrel domain. The 402 amino acids that were identified in the Neandertal genome share a high degree of identity with the corresponding residues of hALOX5. Only 6 amino acids were different, indicating an amino acid identity of 99%. Neither of these differences could be confirmed in a second fossil specimen; therefore, all of them are unconfirmed (+, green background).

Among the iron ligands of hALOX5, the three His are conserved in nALOX5, but there are no sequence data for the other two iron ligands (Fig. 4).

The triad concept of positional specificity is applicable for human ALOX5 (44), as sequential introduction of space-filling amino acids at the triad determinants converted this enzyme gradually to 15-lipoxygenating enzyme species. Similar data have recently been published for mouse Alox5 (45). Here we looked for conservation of the triad residues and found that F360 of hALOX5 is conserved in nALOX5. Unfortunately, there are no sequence data for the other triad determinants in nALOX5.

hALOX5 is a S-LOX and is consistent with the Coffa concept (30), as it carries an Ala at position 411. This Ala is conserved in nALOX5, suggesting S-enantioselectivity of the Neandertal enzyme.

When leukocytes are activated, ALOX5 translocates from the cytosol to intracellular membranes, and calcium is required for membrane binding (46). In the N-terminal β-barrel domain of hALOX5, calcium-binding residues (N44, D45, and E47) and membrane-inserting amino acids (W13, W75, and W102) have been identified; site-directed mutagenesis of these residues impaired membrane binding of the enzyme (47). Unfortunately, none of these residues was specified in nALOX5, so no prediction can be made on its membrane-binding capacity.

The substrate-binding pocket of hALOX5 is an elongated cavity (48, 49), which appears to be sealed at both ends. The bulky side-chains of F178 and Y182, which are not present at this position in rALOX15, close the cavity at one end (FY-cork). The opposite opening may be blocked by the side chains of W148, and thus, substrate penetration appears to be sterically hindered. Two mechanisms have been suggested by which arachidonic acid can enter the active site: i) movement of the FY-cork (decorking) and ii) rotation of the Trp147 side chain. These amino acids are well conserved in nALOX5 (Fig. 4); and therefore, similar mechanisms of substrate binding may be predicted for this enzyme.

When human leukocytes are activated, ALOX5 is phosphorylated by the activity of different protein kinases; three potential phosphorylation sites (S272, S524, and S664) have been identified (50). Phosphorylation of S664 is catalyzed by ERK2 and apparently activates the enzyme (51). Phosphorylation of S272 inhibits nuclear export of hALOX5 (52), and in contrast, phosphorylation of S524 inhibits nuclear import (53). S272 of hALOX5 was conserved in nALOX5, but the other potential phosphorylation sites were not specified.
ALOX12B (12R-LOX)

ALOX12B was first discovered in human (54) and mouse skin (55). Amino acid alignment of hALOX12B and nALOX12B indicated that from the 701 amino acids of hALOX12B, 524 (75%) were identified in the Neandertal genome. Assuming that the number of amino acid residues in hALOX12B and nALOX12B is identical, 177 amino acids (25%) could not be identified in nALOX12B (Table 1). Only about 5% of the unidentified amino acids are localized in the N-terminal β-barrel domain. The 524 amino acids, which were identified in the Neandertal genome, share a high degree of identity with the corresponding residues of hALOX12B. Only 10 amino acids were different, indicating an identity degree of about 98%. Among them, neither difference was confirmed (Table 1), and one difference (G323S) was localized at the beginning of a sequence fragment (Fig. 5). Thus, it was classified as uncertain (+, yellow background). The remaining nine differences (C57R, Y314H, V435I, M467I, D524G, P531L, V570I, H647Y, and P649L) are unconfirmed (++, green background) and are mainly localized in the C-terminal part of the catalytic domain.

Except for the N-terminal Ile, the putative iron ligands of hALOX12B were conserved in nALOX12B, suggesting a functional iron ligand sphere. In hALOX12B, a small Gly represents the enantioselectivity determinant (G441), which is consistent with the Gly-vs-Ala concept (30). As indicated in Fig. 5, this Gly is conserved in the nALOX12B sequence, and thus, the enzyme should be classified as R-LOX. Although the triad determinants are not of major functional importance for mouse Alox12b (36), the corresponding residues present in hALOX12B are also conserved in nALOX12B.

ALOX3 (eLOX3)

ALOX3 was first discovered in mouse and human skin (56, 57), and alternative transcripts have been identified. For initial comparison (Fig. 6A), we first selected the transcript that encodes a β-barrel domain, which is comparable in size with that of the other mammalian LOX isoforms. Amino acid alignment of this hALOX3 isoform 2 and the corresponding Neandertal sequence (nALOX3) indicated that from the 711 amino acids of hALOX3, 522 (73%) were identified in the Neandertal genome. Assuming that the number of amino acid residues in hALOX3 and nALOX3 is identical, 189 amino acids (27%) could not be identified in nALOX3 (Table 1). About 14% of the unidentified amino acids are localized in the N-terminal β-barrel domain. The 522 amino acids, which were identified in the Neandertal genome, share a lower degree of sequence identity (97%) with the corresponding residues of hALOX3 (Table 1). We found 18 amino acid differences, and one of them (H403Y) was confirmed in two different fossils.
Finally, we compared the sequences of the larger transcript (Fig. 6B). Here we found two additional conservative amino acid exchanges (V47I and R48K) as well as a single nucleotide exchange, which introduced a premature stop codon (Q95Stop). These additional alterations were unconfirmed but were localized in the middle of a sequenced fragment (+, green background).

Fig. 5. Dual amino acid alignment of hALOX12B and nALOX12B. The amino acid sequences of the two LOX isoforms were aligned with the DiAlign software. The amino acids, which have not been specified in the Neandertal genome, are indicated by X on gray background. Color coding is explained in the Fig. 1 legend.

fossil specimens (Vi33.16 and Vi33.26); although they are localized at the end/beginning of a sequenced fragment, it must be labeled confirmed (+++, blue background). Among the remaining 17 differences, 8 exchanges (P192F, I290T, C338Y, Q343Stop, Q344Stop, P439S, E559K, and A667T) were identified only in one fossil specimen (+++, green background). Nine amino acid exchanges (A16V, W82Stop, S125F, T217M, R342C, P377L, W388Stop, S560N, and G692N) were classified as uncertain (+, yellow background) because they were localized at the end or the beginning of a sequenced fragment (Fig. 6A). Interestingly, 2 of the unconfirmed (+, green background) nucleotide exchanges (Q343Stop and Q344Stop) introduced premature stop codons, which lead to interruption of the open reading frame. It should be stressed at this point that these differences are caused on the genomic level by two C>T exchanges, which converted the triplets CAG encoding for Gln to TAG stop codons (amber). This means that the 2 identified nucleotide exchanges (-CAG-CAG- in hALOXE3 modified to -TAG-TAG- in nALOXE3) are separated by two well-defined nucleotides (AG), which makes a sequencing artifact less likely.

The putative iron ligands of hALOXE3 are conserved in nALOXE3, but the enantioselectivity determinant (A451 in hALOXE3), which appears to be functional in this LOX isoform (58), was not specified for nALOXE3. Among the triad determinants, only V465 and T466 in hALOXE3 are conserved in nALOXE3, whereas the counterparts of F400 and L641 (hALOXE3) were not specified in nALOXE3 (Fig. 6A).

Finally, we compared the sequences of the larger transcript (Fig. 6B). Here we found two additional conservative amino acid exchanges (V47I and R48K) as well as a single nucleotide exchange, which introduced a premature stop codon (Q95Stop). These additional alterations were unconfirmed but were localized in the middle of a sequenced fragment (+, green background).

**ALOX12E (eLOX12)**

In the LOX cluster on chromosome 11 of mice, there is a functional gene encoding for an epidermis-type 12-LOX. In humans, this gene is present as functionless pseudogene (1), which lacks exonic sequences and also contains premature stop codons. In the Neandertal genome, the corresponding gene is also present as functionless pseudogene (data not shown).

**DISCUSSION**

Comparison of the genomes of ancient hominids with that of today's modern humans offers an alternative approach to explore human evolution that is complementary to morphological studies. In addition, such sequence data will also contribute to answer the philosophical question of what actually makes a modern human being (15). Two years ago, a draft sequence of the Neandertal genome (1.3-fold genome coverage) was published (19), and comparison of the genome sequence of H. neanderthalensis with H. sapiens indicated that the two hominids share common
Fig. 6. Dual amino acid alignment of hALOXE3 and nALOXE3. The amino acid sequences of the two LOX isoforms were aligned with the DiAlign software. The amino acids, which have not been specified in the Neandertal genome, are indicated by X on gray background. Color coding is explained in the Fig. 1 legend. Note the occurrence of two adjacent premature stop codons that are indicated by “–” on green background. (A) ALOXE3 isoform 2. (B) ALOXE3 isoform 1.
ancestors and diverged from each other during evolution about 800,000 years ago. Moreover, the data suggested that the split between the two hominid populations occurred 350,000 years ago and that Neandertals shared more genetic variants with today’s humans in Eurasia than with present-day humans in sub-Saharan Africa. Several months later, the genome of an unrelated archaic hominid (Denisovans) was completed, and the sequence data indicate that Denisovans share a common origin with Neandertals (59).

Comparison of the three genomes (Neandertals, Denisovans and today’s humans) indicated that modern humans living outside Africa carry about 2.5% of their DNA from Neandertals. In contrast, people living today in Australia and New Guinea (Australasians) carry about 5% of Denisovan DNA in their genome. In other words, Neandertals shared more genetic variants with present-day Eurasians than with today’s sub-Sahara Africans, suggesting that gene flow from Neandertals into the ancestors of non-Africans occurred before the divergence of the Eurasian groups from each other. Furthermore, it was concluded that Denisovians were not involved in the gene flow from Neandertals into today’s Eurasians but that they contributed 4–6% to the genome of present-day Melanesians. The most likely explanation for the exchange of genetic material is interbreeding. Modern humans, who left Africa heading for Eurasia about 60,000 years ago, have apparently met Neandertals. Similarly, the ancestors of Australasians may have met Denisovans for genetic exchange. In short, people living outside Africa carry distinct remnants of archaic DNA from such interbreeding events.

Human ALOX15 exhibits a dual positional specificity, converting arachidonic acid to 12S-HpETE and 15S-HpETE in a ratio of about 10:1 (25, 26), but the mouse ortholog mainly produces 12S-HpETE. The structural basis for this difference has been explored (28, 29). Sequence comparison of ALOX15 genes of higher nonhuman primates suggested that rhesus monkeys, similar to mice, exhibit a 12-lipoxygenating ALOX15, and expression studies confirmed this suggestion (38). In contrast, higher nonhuman primates, such as orangutans (38) and gorillas, as well as today’s humans express a 15-lipoxygenating enzyme. These data suggest that a switch between 12- and 15-lipoxygenating ALOX15 species must have occurred during evolution between rhesus monkeys and orangutans. Consistent with this concept is our conclusion that H. neanderthalensis must have expressed a 15-lipoxygenating ALOX15.

Comparison of the genome sequences of ancient hominids with those of today’s humans allowed identification of sequences that are unique to present-day humans. In fact, a number of genomic regions have been identified that may have been affected by positive selection in ancestral modern humans (19), which include genes involved in metabolism (FAAH1, PUR8, and TKTL1), immune modulation (NLRC1, BTLA, and LTK), cognitive functions (ORIK1, O52W1, and ORSK4), and cell signaling (CALD1 and SSH2). Among these sequences was the CLTR2 gene encoding for a cysteinyl leukotriene receptor. Here a F50V exchange was predicted, and although functional studies have not been carried out, the Grantham score of 50 suggests functional relevance of this mutation (19). These data suggest that Neandertals might have suffered from compromised leukotriene signaling.

To follow this line of evidence, we wondered whether the biosynthetic cascade of leukotrienes might also have been compromised. To answer this question, we compared the functional LOX genes of H. sapiens with the corresponding sequences of Neandertal individuals and found that all functional LOX genes detected in H. sapiens (ALOX15, ALOX15B, ALOX12, ALOX12B, ALOX5, and ALOXE3) are conserved in the Neandertal genome. Unfortunately, the sequences are incomplete, which makes evaluation of gene functionality somewhat complicated. On the amino acid level, about 60% (ALOX5) to 75% (ALOX12B) of the LOX amino acid sequences were identified for the different nALOX isoforms; among the identified amino acids, the degree of identity to the H. sapiens LOXs varied between 96 and 99%. If identified, most of the functional amino acids, such as the protein iron ligands, the reaction-specificity determinants (triad determinants and enantioselectivity determinant), the calcium and ATP-binding sites of ALOX5, the membrane-binding determinants, and the potential phosphorylation sites are well conserved, suggesting that the corresponding enzyme properties of hALOX isoforms were conserved in Neandertal lipoxygenases. When we mutated selected amino acid exchanges in hALOX15 and hALOX12, we did not find major alterations in catalytic activities or in reaction specificity, indicating the conservative character of these amino acid exchanges.

In the sequences of nALOX12 and nALOX3, we identified premature stop codons, which interrupt the open reading frame. However, this finding has to be interpreted with care because on the nucleic acid level the mutations are indicated by a C-to-T exchange (a cytosine is present in the human isoforms). Cytosine (C) and thymine (T) are structurally closely related and may be interconverted by deamination during postmortem aging of bones (diagenesis). It may well be that a C was present at these positions in living Neandertal individuals but that these C became deaminated over the years of fossilization. This may also apply to those coding mutations, which are due to a C>T exchange on the nucleic acid level.

Despite these uncertainties, sequence comparisons suggest that ALOX15, ALOX5, ALOX15B, and ALOX12B should have been present as functional proteins in H. neanderthalensis, whereas expression of ALOXE3 and ALOX12 might have been compromised. ALOXE3 has been implicated in skin barrier function (60) and adipocyte development (61), and mutation of the human gene was associated with a special type of ichthyosis, a congenital disease characterized by a scaly, fish-like skin. Whether Neandertals have suffered from evolutionary disadvantage owing to dysregulation of epidermal differentiation remains to be explored. The biological role of ALOX12 has been difficult to decipher. Alox12 knockout mice are viable and breed normally (11). ADP-induced aggregation of Alox12-deficient platelets is somewhat compromised (11), and in
the skin, the enzyme has been implicated in maintenance of the water permeability barrier (62). Here again, it remains unclear of whether any of these defects may have been relevant for the physiology of Neandertals.

The currently available sequence of the Neandertal genome (19) constitutes an excellent tool for investigating the evolutionary relationship between different ancient hominids and modern human beings that left Africa 60,000 years ago. Moreover, the data suggest some candidate genes for positive selection early on in modern human history. However, it is rather problematic to conclude the functionality of specific gene products on the basis of these sequences for two reasons. First, the genome sequence is incomplete, and for the coding region of nALOX genes, only 60–75% of the amino acids have been specified. Thus, it may still be possible that peculiarities in nALOX genes have escaped detection because of lacking sequence data. Second, the currently available genomic sequences are based on sequence data obtained from three different fossil specimens (19), and most of these sequences are not overlapping. Thus, it remains unclear at the moment whether the observed differences to the *H. sapiens* genome are conserved in other Neandertal individuals. It might well be that some of the differences are caused by single nucleotide polymorphisms, which are specific for the sequenced Neandertal individuals. Future analyses of other Neandertal fossils are likely to put the functional conclusions on a more profound basis. 

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