Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 485–489, January 1992
Biochemistry

Mutagenesis of some conserved residues in human 5-lipoxygenase: Effects on enzyme activity

(leukotrienes/eicosanoids/arachidonic acid/dioxygenase)

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Contributed by Bengt Samuelsson, September 23, 1991

ABSTRACT **Recombinant human 5-lipoxygenase (arachidonate:oxygen 5-oxidoreductase, EC 1.13.11.34) was expressed in *Escherichia coli*. In incubations of *E. coli* supernatants with arachidonic acid, 5-hydroxy-7,9,11,14-eicosatetraenoic acid and leukotriene A_4 were formed, while incubation with 8,11,14-eicosatrienoic acid gave 8-hydroxy-9,11,14-eicosatrienoic acid. Six conserved histidine residues in 5-lipoxygenase were subjected to site-directed mutagenesis. Exchanges of His-367, -372, or -551 gave mutants for which no enzyme activities were detectable. On the other hand, exchanges of His-362, -390, or -399 gave mutants that were enzymatically active, but less so than the nonmutated control. For two of these (exchanges of His-390 or -399), the activities of the mutants were dependent on the expression temperature. Thus, the histidines in the first group (His-367, -372, -551) were crucial for 5-lipoxygenase activity, possibly because of a function of these residues as metal ligands. Mutagenesis aimed at two other conserved elements in 5-lipoxygenase, Gln-558 and the C terminus, gave mutated proteins with only a small residual activity (substitution of Gln-558), or with no detectable activity (deletion of six C-terminal amino acids), indicating that these regions are important for the function of 5-lipoxygenase.**

5-Lipoxygenase (5LO; EC 1.13.11.34) catalyzes oxygenation of arachidonic acid at C-5, leading to 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE), and the further conversion of 5-HPETE to leukotriene A_4 (LTA_4) (1). 5LO has been purified from human leukocytes and other phagocytic cells (2). Also, cDNAs for human and rat 5LO (3–5), and the human 5LO gene (6), have been cloned. Recombinant human 5LO has been expressed in mammalian osteosarcoma cells (7), in insect cells (8, 9), in bacteria (10), and in yeast (11).

cDNA sequences have also been determined for four other mammalian lipoxygenases and for four plant lipoxygenases: 15LO from human and rabbit reticulocytes (12, 13), 12LO from porcine leukocytes (14) and human platelets (15, 16), three isozymes of soybean lipoxygenase (17–19), and a pea lipoxygenase (20). Upon comparison of the 10 lipoxygenase amino acid sequences, some conserved features have been obvious. Most recognized is a set of five histidine residues, which are equally distributed in all lipoxygenases (at positions 362, 367, 372, 390, and 399 in human 5LO). A sixth conserved histidine lies further toward the C terminus (His-551 in 5LO).

Soybean lipoxygenase contains 1 mol of nonheme iron per mol of protein, which shuttles between the Fe(II) and the Fe(III) states during catalysis (21). The iron is probably six-coordinate (22) and it was found that the metal is bound via 4 ± 1 nitrogen ligands (imidazole) and 2 ± 1 oxygen ligands (carboxylate) (23, 24). The ferric form of the enzyme (but not the ferrous) may have one exchangeable water ligand (25, 26), and it has been suggested that the coordination sphere of soybean lipoxygenase might change upon oxidation and substrate binding (27). Recombinant 5LO was reported to contain 1.1 mol of iron per mol of protein, and the iron was lost upon treatment with oxygen, which also inactivated the enzyme (28).

The six conserved histidine residues have been implicated as possible metal ligands in lipoxygenases and may thus be important for enzyme activity. We have determined the effects of mutagenesis of these histidines on the enzyme activity of recombinant 5LO expressed in *Escherichia coli*. Also, the effects of mutations of two other conserved elements (Gln-558 and the C terminus) were investigated.

MATERIALS AND METHODS

Construction of Mutagenesis and Expression Plasmid pT3-5LO. The same vector was used for both mutagenesis and expression of 5LO. A 5LO cDNA fragment was excised from the plasmid pERAT308-pl5BSm (8) with *Eco*RI and *Bcl* I. The fragment contains (from the 5′ end) an *Eco*RI site, 34 base pairs (bp) of noncoding sequence, the entire coding region, 164 bp of 3′ noncoding sequence, and finally the *Bcl* I site at the 3′ end. In a procedure involving several steps, the 34-bp 5′ noncoding sequence was deleted by oligonucleotide-directed loop-out mutagenesis, and the 3′ end was changed to a *Sal* I site. The resulting fragment was then inserted into the polylinker site of pT7T3 (Pharmacia). In a second loop-out mutagenesis, a 41-bp fragment around the T7 promoter was deleted. In the final plasmid (pT3-5LO, Fig. 1) the ATG initiation triplet is located 10 bp downstream of the Shine–Dalgarno (ribosome binding) sequence.

Mutagenesis. The mutants were generated by oligonucleotide-directed mutagenesis (29) using a Muta-Gene kit from Bio-Rad. Briefly, the single-stranded uracil-containing pT3-5LO cDNA was prepared from a Dut^- Ung^- *E. coli* strain (CJ236) and annealed to a synthetic oligonucleotide including the desired mutation. After elongation and ligation, the double-stranded plasmid was used to transform *E. coli* MV1190 (Dut^+ Ung^+). Propagation in this strain removes the uracil-containing strand.

Mutated clones were identified by sequencing double-stranded DNA with a T7 sequencing kit (Pharmacia); the mutagenesis efficiencies were usually between 25% and 100%. Each mutated cDNA was purified by repeated transformation and cloning, and the authenticity of each mutated cDNA was confirmed by sequencing single-stranded DNA of the entire coding region as well as the *lacZ* promoter region.

Expression. *E. coli* MV1190 was transformed with pT3-5LO and the different mutated DNAs, and overnight cultures

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Abbreviations: 5-H(P)ETE, 5(*S*)-hydro(pero)xy-7-*trans*-9,11,14-*cis*-eicosatetraenoic acid; 5LO, 5-lipoxygenase; LTA_4, leukotriene A_4 [5(*S*),6-oxido-7,9,11-*trans*-14-*cis*-eicosatetraenoic acid]; IPTG, isopropyl β-D-thiogalactopyranoside.

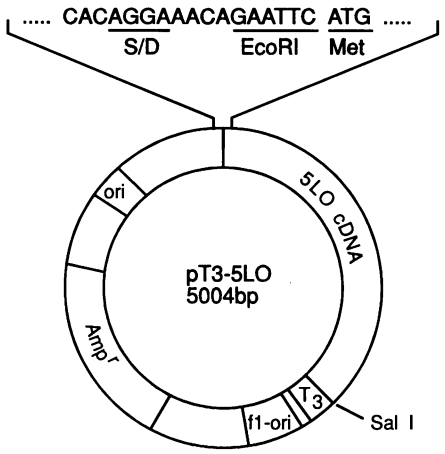

FIG. 1. Map of plasmid pT3-5LO (not exactly to scale).

were prepared. For expression, 10 ml of modified M9CA medium (Na_2HPO_4, 6 mg/ml; KH_2PO_4, 3 mg/ml; NaCl, 0.5 mg/ml; NH_4Cl, 1 mg/ml; $MgSO_4$, 2 mM; glycerol, 20 mg/ml; Casamino acids, 2 mg/ml; ampicillin, 150 μg/ml) was inoculated with 50 μl of overnight culture. Different temperatures were tried for expression (see below). When the cell density reached OD_{620} of 0.2–0.5, isopropyl β-D-thiogalactopyranoside (IPTG) was added to 100 μM. At OD_{620} of $\approx$2 the cells were harvested. The collected *E. coli* pellets were washed with 50 mM triethanolamine/HCl (pH 7.5), containing 100 mM NaCl and 5 mM EDTA. After washing, the pellets were snap-frozen and kept at −20°C until use.

Assay of 5LO Enzyme Activity. The frozen *E. coli* pellets were resuspended in sonication buffer [50 mM Tris Cl, pH 8.0/5 mM EDTA/2 mM dithiothreitol with soybean trypsin inhibitor (60 μg/ml) and lysozyme (500 μg/ml)] to an OD of about 20. After 30 min of incubation on ice, the suspensions were sonicated on ice with an MSE mkII 150-W ultrasonic disintegrator (three bursts of 5–8 sec each, amplitude 18). The homogenates were centrifuged at 15,000 × *g* for 15 min at 4°C.

The assay mixture (100 μl) contained 160 μM arachidonic acid, 9 μM 13-hydroperoxy-9,11-octadecadienoic acid (activator and internal standard), 1 mM ATP, 5 mM $CaCl_2$, 50 mM Tris Cl (pH 7.5), and various volumes of the 15,000 × *g* supernatant. When the 100,000 × *g* supernatant was used, phosphatidylcholine vesicles (20 μg/ml, produced by sonication) were also included in the assay mixture. Incubation was at room temperature for 10 min and was stopped by addition of stop solution: A [200 μl of MeCN/MeOH/HOAc (1:1:0.002, vol/vol)] or B [900 μl of MeOH/0.1 M HCl/H_2O (5:1:3)] and prostaglandin B_2 (600 pmol).

For analysis of the 5-HPETE and 5-HETE production, stop solution A was used. The mixture was vortex-mixed and centrifuged at 15,000 × *g* for 10 min to remove precipitates. An aliquot of the supernatant was injected on a Nova-Pak C_{18} HPLC column (Waters) and eluted isocratically with MeCN/MeOH/H_2O/HOAc (1:1:1:0.002) at 1.2 ml/min. The eluent was monitored at 234 nm, and the sum of 5-HPETE plus 5-HETE formed was calculated using the sum of the 13-hydroperoxy/hydroxy-9,11-octadecadienoic acid peaks as internal standard. Usually, the peaks corresponding to 5-HETE and 13-hydroxy-9,11-octadecadienoic acid were predominant in the chromatograms, indicating a considerable peroxidase activity in the *E. coli* extracts.

For estimation of LTA_4 production as well, incubations were stopped with solution B. After removal of the precipitate, the lipids were extracted on a Chromabond C_{18} column (Macherey & Nagel). The column was washed with 2 ml of MeOH and 2 ml of H_2O, and the acidified sample was applied. Impurities were removed with 2 ml of H_2O and 2 ml of MeOH/H_2O (1:3), and finally the sample was eluted with 2 ml of MeOH. The MeOH was evaporated under nitrogen at 40°C. The lipids were then taken up in 0.16 ml of 50% MeOH and injected onto the Nova-Pak C_{18} column equilibrated with MeCN/MeOH/H_2O/HOAc (1:1:1:0.002). The separation was monitored at 234 nm for 5-HPETE/5-HETE and at 270 nm for LTA_4 derivatives. Prostaglandin B_2 served as internal standard for estimating the quantity of LTA_4-derived compounds (including 5,12-diHETEs, 5,6-diHETEs, and the MeOH trapping products).

Protein Assay. Protein was determined (30) by Bio-Rad protein assay with bovine gamma globulin as standard.

Western Blot Analysis. The proteins were separated by SDS/PAGE, transferred to nitrocellulose membrane (Amersham Hybond-C), and incubated with rabbit anti-5LO antiserum (3) followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma). The immunoreactive proteins were visualized by immersion in a solution of 25 mg of fast red TR salt and 10 mg of α-naphthyl disodium salt in 50 ml of 50 mM Tris·HCl, pH 9.0/2 mM $MgCl_2$.

RESULTS AND DISCUSSION

Expression of Nonmutated 5LO in *E. coli*. Recombinant 5LO was expressed in *E. coli* MV1190 transformed with the plasmid pT3-5LO (Fig. 1). In this plasmid, transcription is initiated at the first ATG codon of the 5LO cDNA, and expression is controlled by the *lacZ* promoter.

The time course for the expression of 5LO enzyme activity in poor culture medium (M9CA) was carried out at 18°C and at 37°C (Fig. 2). IPTG (0.1 mM) was added when the cell density had reached $OD_{620} \approx 0.2$, and the cultures were continued up to OD $\approx$ 4. For both temperatures, the IPTG-induced expression of 5LO enzyme activity was parallel to the increase of cell density, and the amounts of active enzyme obtained were about the same at 18°C (after 36 hr) and at 37°C (after 11.5 hr). This was not expected, since previous reports regarding expression in *E. coli* of human 5LO (10) and of a rice lipoxygenase (31) stated that culture at 37°C mainly gave inactive enzymes sequestered in inclusion bodies.

Expression at 18°C was chosen as the routine condition for investigation of the enzyme activity of 5LO mutants. However, all mutants were also expressed at 37°C, and for some the yield of active enzyme was temperature-dependent (see below).

Arachidonic acid was the usual substrate for assay of enzyme activity, and the amount of 5-HPETE plus 5-HETE formed was estimated by HPLC. Some samples were assayed also for the formation of LTA_4. For the nonmutated recombinant 5LO, the formation of LTA_4 was 15% of the formation of 5-HETE when incubated with arachidonic acid, as found for human leukocyte 5LO (2). The conversion of exogenous 5-HPETE to LTA_4 could not be examined, because of peroxidase activity of the *E. coli* supernatants.

8,11,14-Eicosatrienoic acid is another substrate for 5LO (32–34); oxygenation at C-8 gives 8-hydroxy-9,11,14-eicosatrienoic acid. This reaction is mechanistically similar to the conversion of 5-HPETE to LTA_4 (28), in that both the position and stereochemistry of the hydrogen abstractions are the same. The recombinant human 5LO catalyzed the conversion of 8,11,14-eicosatrienoic acid (with an efficiency that was about 20% of the conversion of arachidonic acid), as previously shown for 5LO from potato tubers and mouse mastocytoma cells (33, 34).

The effect of addition of iron to the expression medium was investigated. At 5 μM, ferrous ammonium sulfate had no effect on the expression of 5LO activity, whereas ferrous sulfate and ferric nitrate gave 30% and 15% more activity,

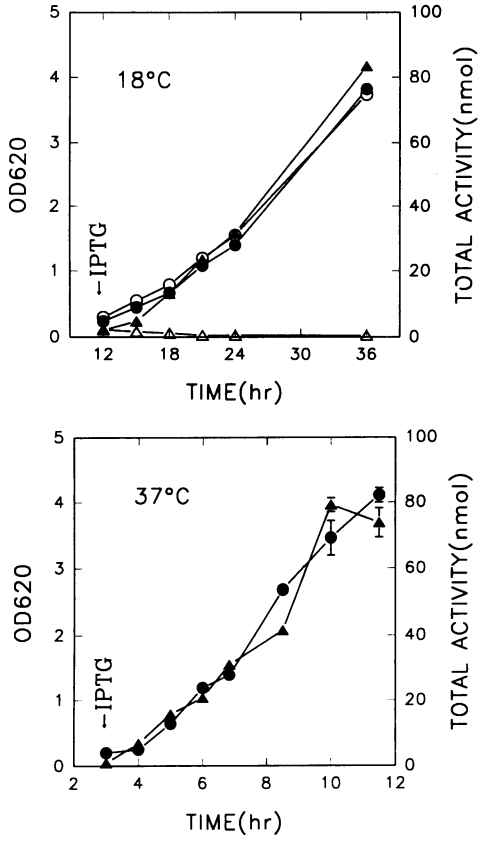

FIG. 2. Time course of expression of 5LO. *E. coli* MV1190 transformed with pT3-5LO was grown at 18°C (*Upper*) or 37°C (*Lower*). At each time point, a 10-ml aliquot was removed from the culture. Cell densities (circles, OD_{620}) and 5LO activities (triangles) were determined for aliquots (60 μl) of the 15,000 × *g* supernatant of each *E. coli* homogenate (see *Materials and Methods*). The total activities in the 10-ml aliquots were plotted. Filled and open symbols represent expression cultures with and without IPTG induction, respectively. Each point represents the average from two different cultures; the deviations from the mean values are indicated.

respectively. The addition of iron gave a more pronounced effect on one of the mutants (see below).

5LO Mutants. Ten mutants of human 5LO were constructed, 8 of which concerned conserved histidine residues (Table 1). The sequence of the entire coding region and the promoter was determined for each mutant, and the 5LO enzyme activity was determined after transformation of *E. coli* with single-stranded DNA from the sequenced pool.

In a representative experiment (Table 2), cells transformed with pT7T3 (lacking a cDNA insert) gave no detectable activity, whereas transformation with pT3-5LO gave an enzyme activity (in the 15,000 × *g* supernatant) of 21.8 nmol/mg, after IPTG-induced expression at 18°C for 12 hr.

The mutations of the conserved histidine residues gave results that could be divided in two groups, one with completely abolished enzyme activity (H367Q, H372Q, H372S, H551L, and H551Q), another with reduced enzyme activity (H362Q, H390Q, and H399Q).

Mutations of His-367, -372, or -551. For mutants in the first group, in all determinations enzyme activity was undetectable. The same samples were assayed for 5LO immunoreactivity on Western blots (Fig. 3), indicating that the amounts of mutated proteins (H367Q, H372Q, H372S, H551L, and H551Q) were similar to that of nonmutated recombinant 5LO in the positive control. This is based on the assumption that all proteins (mutated or not) were equally immunoreactive. We conclude that His-367, His-372, and His-551 are crucial for the enzyme activity of 5LO and may represent three of the metal ligands (see below). His-367 and -372 are included in the stretch 367–381, which is homologous with the interface-binding domain of human lipoprotein lipase (4). This region has been suggested to be involved in substrate binding. Thus, it is possible that mutations of His-367 and His-372 could interfere with several aspects of 5LO function.

Table 1. 5LO mutants

Name	Codon change	Change in protein
H360Q	CAC → CAG	His-360 → Gln
H362Q	CAC → CAG	His-362 → Gln
H367Q	CAC → CAG	His-367 → Gln
H372S	CAT → TCT	His-372 → Ser
H372Q	CAT → CAG	His-372 → Gln
H390Q	CAC → CAG	His-390 → Gln
H399Q	CAC → CAG	His-399 → Gln
H551L	CAC → CTC	His-551 → Leu
H551Q	CAC → CAA	His-551 → Gln
Q558E	CAG → GAG	Gln-558 → Glu
C6	Nucleotides 2005–2022 deleted	C-terminal six residues deleted

A different result has been published from this laboratory regarding the mutant H372S, which was found to have the same enzyme activity as nonmutated 5LO, when expressed in insect cells (8). Possibly, these controversial findings could be due to different capacities for posttranslational modifications and protein folding in insect cells versus *E. coli*. With the *E. coli* expression system, however, both the H372S and H372Q enzymes were inactive, and the DNA sequences of the actual expression plasmids could be determined.

Mutations of His-362, -390, -399, or -360. Mutations of the additional conserved histidines (at positions 362, 390, and 399) gave proteins with partial 5LO enzyme activity. In different experiments there was some variation in the activities of these mutants in relation to the positive control, probably due to variations in expression levels.

Table 2. Enzyme activities of 5LO mutants

Cells*	Activity, nmol/mg		
	5-H(P)ETE†	LTA_4‡	8-H(P)ETrE†
Negative	0	0	0
Normal	21.8	3.2 (15%)	5.2
H362Q	7.2	0.2 (3%)	0.02
H367Q	0	0	0
H372S	0	0	0
H372Q	0	0	0
H390Q	5.4	0.4 (7%)	0.08
H399Q	15.9	1.4 (9%)	1.5
H551L	0	0	0
H551Q	0	0	0
Q558E	0.7	0	0
C6	0	0	0

E. coli cells were transformed with mutated single-stranded DNAs. For each mutation, two clones were tested. The expression temperature was 18°C, and the cultures were grown for 12 hr after addition of IPTG (100 μM). The enzyme activities were determined by incubations (10 min, room temperature) of the 15,000 × *g* supernatants with 160 μM arachidonic acid [for 5-H(P)ETE and LTA_4] or 8,11,14-eicosatrienoic acid [for 8-hydro(pero)xy-9-*trans*-11,14-*cis*-eicosatrienoic acid, H(P)ETrE].

*Negative, *E. coli* transformed with pT7T3 (lacking a cDNA insert); normal, *E. coli* transformed with pT3-5LO.

†Sum of hydroperoxy and hydroxy derivatives. Each number is the average of results from two clones.

‡Determined as the sum of the hydrolysis products and the MeOH trapping products of LTA_4. Values in parentheses are LTA_4/H(P)ETE ratios.

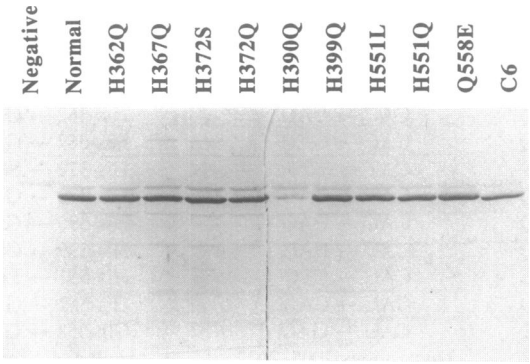

FIG. 3. Western blot analysis of mutated proteins. For each mutation, one of the clones expressed (see Table 2) was also analyzed for 5LO immunoreactivity. Aliquots of the 15,000 × *g* supernatants (60 μg of protein) were applied to an SDS/polyacrylamide gel. Western blotting and immunostaining were as described in *Materials and Methods*. Negative and normal, see Table 2.

The mutant H362Q gave enzyme activity that was about 10–30% of the activity of the positive control, although the amount of mutated protein was similar to the control. There was no clear effect of expression temperature on the activity of this mutant. Previously, the mutant H362S was found to have the same activity as nonmutated 5LO, when expressed in insect cells (8). It thus appears that His-362 may not be absolutely required for 5LO enzyme activity.

Mutations at His-390 or -399 gave proteins with 5LO activity that was dependent of the expression temperature. The mutant H399Q gave 60–80% of the total activity of the positive control after expression at 18°C. The corresponding immunoblots appeared compatible with the activity data, in analyses both of 15,000 × *g* supernatants (Table 2; Fig. 3) and of 100,000 × *g* supernatants (Table 3; Fig. 4). However, the activity of H399Q was <2% of the control after expression at 37°C (Table 3), although about the same amount of protein was formed as at 18°C (Fig. 4). The mutant H390Q gave 15–25% of the total activity of the control after expression at 18°C, together with a clearly decreased Western blot signal. After expression at 37°C the activity was about 1% of the control, but more protein was formed than at 18°C. The markedly decreased immunoblot signal for the mutant H390Q (Fig. 3) could indicate a low immunoreactivity of this mutated protein. Alternatively, only small amounts of H390Q protein were formed at 18°C, and the data in Tables 2 and 3 underestimate the activity of this mutant.

The reason for the temperature sensitivity of these mutants is unclear, but two suggestions are given. (*i*) If His-390 or -399 is involved in metal binding, it is possible that Gln could substitute as a ligand, albeit less efficiently, at these particular positions. The putative weaker association between Gln and metal would thus be stabilized at decreased temperature. (*ii*) Alternatively, these histidines are not involved in metal binding, and the explanation would be that Gln-390 or -399 is compatible with correct protein folding only at the lower temperature. When one of these mutants (H399Q) was expressed at 37°C with addition of ferrous sulfate (5 μM) to the culture medium, a 4.5-fold increase in 5LO activity was obtained. This was not the case for H390Q, which may indicate that positions 390 and 399 are of different significance for iron binding. Additional mutations at these positions, to amino acids definitely incapable as ligands, should clarify the involvement of His-390 and -399 in metal binding.

Replacement of His-360 (which is conserved only in the 5LOs) by Gln gave only a slight reduction in activity, to 79% of the control after expression at 37°C, and to 85% of the control after expression at 18°C. For this mutant only the formation of 5-HETE was determined.

Table 3. Comparison of enzyme activities for certain mutants expressed at 18°C and at 37°C

Cells*	5-H(P)ETE, nmol/mg		37°C/18°C ratio
	18°C	37°C	
Negative	0	0	
Normal	18.1	17.0	0.94
H362Q	2.0	3.1	1.55
H390Q	1.3	0.1	0.08
H399Q	10.2	0.2	0.02

E. coli cells were transformed with mutated single-stranded DNAs. For each mutation, two clones were expressed at both 18°C and 37°C. After addition of IPTG (100 μM), the cultures at 18°C were grown for 14.5 hr (OD_{620} 1.3–1.6), and the cultures at 37°C were grown for 4 hr (OD_{620} 2.2–2.4). Enzyme activities were determined by incubations (10 min, room temperature) of the 100,000 × *g* supernatants with 160 μM arachidonic acid. Each number is the average of the results from two clones.
*Negative and normal, see Table 2.

Mutation of Gln-558. Both His-551 and Gln-558 are located in a region with high degree of similarity among the different lipoxygenases. For example, when residues 546–559 of 5LO are compared with the corresponding residues of the plant lipoxygenases, 12 or 13 out of 14 residues are identical (4). When the region 541–569 of 5LO is compared with the other mammalian 12LOs and 15LOs, 13–17 of 29 residues are identical. Apart from His-551 (see above), Gly-557 and Gln-558 are found in all 10 enzymes. Replacement of Gln-558 by Glu almost completely eliminated the enzyme activity when expressed at 18°C (Table 1) or at 37°C. It thus appears that Gln-558 is important for some functional aspect of 5LO. Alternatively, introduction of a negative charge in this supposedly important part of the enzyme disturbed the three-dimensional structure sufficiently to give an inactive protein. Possibly this conserved region is part of (or close to) the active site.

Deletion of C terminus of 5LO. Human and rat 5LOs are 93% identical; however, their last 7 amino acids are different. On the other hand, human 5LO is only 28–31% identical to soybean and pea lipoxygenases, while 5 out of 7 residues are identical in their C termini. Also, when human 5LO is compared with 15LO (human and rabbit reticulocyte) or

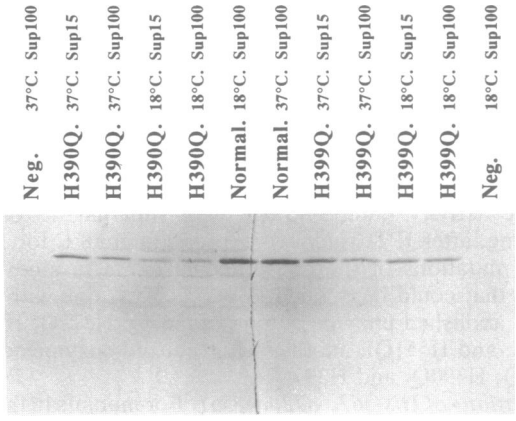

FIG. 4. Western blot analysis of mutated proteins: Temperature-sensitive mutants. For the mutants H390Q and H399Q, one of the clones expressed (see Table 3) was also analyzed for 5LO immunoreactivity. For each clone, aliquots (60 μg of protein) of 100,000 × *g* (Sup100) as well as 15,000 × *g* (Sup15) supernatants were subjected to SDS/PAGE followed by Western blot analysis. For this membrane, a 5LO antiserum that had been adsorbed with *E. coli* proteins was used. Negative (Neg.) and normal, see Table 2.

12LO (human platelet and porcine leukocyte), 5 out of 7 C-terminal amino acids are the same.

When the 6 C-terminal amino acids of human 5LO were deleted, the resulting protein had no enzyme activity (mutant C6; Table 1). For mutant C6, it was observed in several of the expression series that smaller amounts of the mutated protein were obtained (although not as severe a reduction as for the mutant H390Q). This could indicate a decreased immunoreactivity or possibly the C-terminal amino acids are important for the stability of the 5LO protein as well as for conformation and enzyme activity.

Effects of Mutations on LTA_4 Synthase and 8-Lipoxygenase Activities of 5LO. Nonmutated recombinant 5LO catalyzed the formation of LTA_4 and the oxygenation at C-8 of 8,11,14-eicosatrienoic acid (see above), and the mutants were also tested for these enzyme activities. Mutations that were deleterious for conversion of arachidonic acid to 5-HETE (H367Q, H372S, H372Q, H551L, H551Q, Q558E, and C6; see Table 1) also led to complete loss of these other activities. Obviously, when no 5-HETE (and thus no 5-HPETE) is formed, LTA_4 biosynthesis cannot be expected. However, 8LO activity (which is mechanistically similar to LTA_4 synthase activity; see above) was abrogated simultaneously for all these mutants.

The mutants H362Q, H390Q, and H399Q could form the hydrolysis products of LTA_4. The LTA_4/5-HPETE ratio was smaller for these mutants (3–9%; Table 1) than for nonmutated 5LO (15%). This appears to be reasonable, since the mutated enzymes should be less efficient also in converting 5-HPETE to LTA_4. The decrease in LTA_4 synthase activity should be similar to the decrease in 5LO activity for each mutant.

However, the 8LO activity appeared to be more severely affected than the 5LO and LTA_4 synthase activities, particularly for the mutants H362Q and H390Q. Probably, this reflects that although there are mechanistic similarities between these reactions, the binding between enzyme and the different substrates is not exactly identical. These mutations appear to be more deleterious for the binding and conversion of 8,11,14-eicosatrienoic acid.

Conclusions. Recently, recombinant human 5LO was shown to contain 1.1 mol of iron per mol of protein (28). In this study we have investigated the effects on 5LO enzyme activity of mutagenesis of six conserved histidine residues suggested to function as metal ligands in lipoxygenases. Changes of His-367, -372, or -551 gave completely inactive mutants. On the other hand, substitutions at His-362, -390, or -399 gave mutants with enzyme activities that were reduced but clearly detectable. The histidines were primarily replaced with glutamine residues. The hydropathy indexes of histidine and glutamine are quite similar (35), so these substitutions would not be expected to disrupt the protein entirely.

If the metal ligands of 5LO are necessary for enzyme activity, it appears more likely that the histidines in the first group could function as metal ligands than those in the latter. If the spectroscopic findings regarding the iron ligands of soybean lipoxygenase (see Introduction) are valid also for human 5LO, there should be additional imidazole nitrogen ligands (in addition to the putative ligands His-367, -372, and -551). If any of the histidines in the latter group (His-362, -390, and -399) binds iron, it would thus appear that different histidine ligands can have different characteristics. Possibly, they could be of different priority for the association of metal to the protein and for the function of the metal in catalysis.

We thank Drs. Takashi Izumi, Shigeru Hoshiko, and Kamel Darwish for valuable discussions and advice. We thank Ms. Agneta Nordberg for technical assistance. This study was supported by grants from the Swedish Medical Research Council (03X-217 and 03X-7464) and from King Gustaf V's 80-Years Fund.

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