Iron Content of Human 5-Lipoxygenase, Effects of Mutations Regarding Conserved Histidine Residues*

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Recombinant human 5-lipoxygenase was expressed in Escherichia coli and purified to more than 95% homogeneity by ammonium sulfate precipitation and agarose-ATP column chromatography. The specific activity of the purified enzyme was 21–28 μmol/mg, as assessed by the generation of 5-hydro(pero)xyeicosatetraenoic acid. The iron content was analyzed by graphite furnace atomic absorption spectrophotometry for six preparations of the enzyme. The average value of the iron content was 0.86 mol/mol (iron/protein) with a range of 0.74–1.15 mol/mol.

All lipoxygenases that have been sequenced contain 6 conserved histidine residues. Mutants of 5-lipoxygenase, with substitutions of these 6 conserved histidines, were purified and analyzed. Mutants H372Q and H550Q had no detectable enzyme activity and were also practically devoid of iron. Three mutants regarding His367 (H367Q, H367N, and H367S) were all inactive but had partial iron contents (0.5, 0.2, and 0.5 mol/mol, respectively). Finally, the mutated proteins H362Q, H390Q, and H399Q displayed reduced enzyme activity but contained similar amounts of iron as non-mutated 5-lipoxygenase.

We conclude that histidines 372 and 550 constitute two of the iron ligands in 5-lipoxygenase. Also His367 is necessary for the enzyme activity, but this residue is not crucial for binding of iron.

Human 5-lipoxygenase (5-LO) catalyzes the first two reactions in the conversion of arachidonic acid to the leukotrienes (1, 2). These compounds are regarded as mediators of inflammation and hypersensitivity reactions, and the possible pathophysiological roles of the leukotrienes have stimulated intensive research regarding 5-LO and related enzymes (3).

Iron was first found in soybean lipoxygenase (4–6), and subsequently also in mammalian lipoxygenases (7–12). The role for iron in the generally accepted scheme for the lipoxygenase reaction (the radical reaction mechanism) is to act as electron acceptor and donor, during hydrogen abstraction and peroxide formation, for review see Refs. 13 and 14. Also in the alternative "organoiron intermediate" lipoxygenase mechanism, iron has been assigned a central role (15).

Heme or iron-sulfur clusters were not found in soybean lipoxygenase, and it was suggested that the iron should be bound directly to functional groups in the protein (16). From various spectroscopic analyses, the iron in soybean lipoxygenase-1 was reported to be six-coordinate (17), bound by 4 O: 1 nitrogen ligands (imidazole) and 2 O: 1 oxygen ligands (18), in a roughly octahedral field of symmetry (19). The possible involvement of histidine residues in the active site of the enzyme is thus suggested.

Six histidines are conserved in all the cloned lipoxygenases (see references in Ref. 20 and see also Refs. 21–24). In human 5-LO, these histidines are at amino acid positions 362, 367, 372, 390, 399, and 550, when the first methionine is excluded. We apologize here for incorrectly referring to His550 as His551 in our previous report (20).

Mutagenesis studies have been carried out to elucidate the importance of the conserved histidines for lipoxygenase activity (20, 25–27). It was found that substitutions of His367, His372, or His550 (or the corresponding residues in soybean lipoxygenase-1) abolished the enzyme activity completely, whereas replacements of His362, His399, or His550 resulted in enzymes with partial activity.

To determine the iron content, the mutated proteins need to be purified. However, human 5-LO is known to be very unstable after purification (28), and it was found that the enzyme is inactivated when exposed to oxygen, which also caused the release of iron from 5-LO (12). Considering these reports, we modified previously published procedures (29–31) for purification of 5-LO expressed in Escherichia coli. With this method, 5-LO and its various histidine mutants were prepared and subjected to iron content analysis.

MATERIALS AND METHODS

Expression of Recombinant 5-LO and Its Mutants—The construction of the expression vector pT3-5LO and the preparation of various histidine mutants of 5-LO were described in a previous communication (20). The expression plasmid pT7T3 (Pharmacia) was used to transform E. coli for negative control expression cultures.

Two additional mutants (H367N and H367S) were prepared in this study following the mutagenesis procedure described previously (20). The His367 codon CAC was changed to AAG (asparagine), or to TCC (serine), to produce His367N and His367S, respectively.

The procedure used for expression was as described in our previous report (20) except that the culture volume was increased to 1 liter, and the incubation was carried out at 26–28 °C for 18 h. Isopropyl-β-D-thiogalactopyranoside (0.2 mM) was added 6 hrs after the inoculation, and the modified M9CA culture medium contained 5 mM FeSO4.

Purification—All the proteins studied in this report were purified by the same procedure which consisted of two steps: ammonium sulfate precipitation and agarose-ATP (AGATP) column chromatography. E. coli (from 1 liter of culture) were homogenized by sonication (Branson sonicator, three bursts of 15 s each, level 4) in 50 ml of buffer containing 50 mM TEA, pH 8.0, 5 mM EDTA, 2 mM dithio-
threitol, 0.06 mg/ml soybean trypsin inhibitor, 1 mM phenylmethyl-
sulfonyl fluoride, and 0.5 mg/ml lysozyme. The homogenate was
centrifuged at 16,000 × g for 15 min, and the supernatant was
collected (sup16). The 16,000 × g pellet was resuspended in 25 ml of
sorbitol buffer (3 M sorbitol, 1 mM N-ethylmaleimide, and 1 mM
cysteine) and subjected to sonication and centrifugation. The combined
sup16s were mixed with an equal volume of a saturated ammonium sulfate solution and stirred on ice
for 30-40 min. The precipitate was collected by centrifugation at
16,000 × g for 25 min and stored at −20 °C for later use.

Prior to AGATP column chromatography, the ammonium sulfate
pellet was resuspended in 25 ml of buffer A (50 mM TEA, pH 7.3, 2
mM EDTA, 2 mM dithiothreitol, and 10 mM BME) and centrifuged at
100,000 × g for 1 h. The supernatant was collected (sup100) and
loaded directly onto a 1-ml size AGATP column (AGATP, type 3,
Pharmacia) equilibrated with buffer A. The column was connected to a
FPLC system (Pharmacia) and was run at a flow rate of 0.3 ml/ min.
The loading of the sup100 was interrupted (at every 6 ml) by 3-
ml washes (consisting of 1 ml of buffer A, 0.5 ml of linear gradient to
buffer B (1 M NaCl in buffer A), followed by a gradient (0.5 ml) back
to buffer A, and then 1 ml buffer A). After complete loading, 5 ml
each of buffer B and buffer A was applied to the column for two
cycles and then the column was ready for elution.

Since the 5-LO proteins purified from the column were to be used
directly for iron content analysis, an additional cleaning step was
carried out. Every part of the FPLC system which was connected to
the column (sample loop, injection needle, and fill port) was replaced with
a clean set, washed thoroughly with buffer C. Buffer C was prepared by
filtering a solution of 50 mM TEA, pH 7.3, 2 mM dithiothreitol, and 100 mM NaCl through a metal chelating membrane
(Bio-Rad). EDTA (1 mM) was added after the filtration step and then
the buffer was stored at 4 °C. BME (10 mM) was added to an aliquot
of the buffer (0.5 ml) and the loaded column was also cleaned by
washing with 6 ml of the buffer C.

The elution was performed by applying 4 ml of AMP solution (25
mM AMP in buffer C) followed by 5 ml of ATP solution (12 mM ATP
in buffer C, pH 7.3). The ATP and AMP solutions were filtered (100
ml of solution without EDTA and BME) through two chelating membranes with five passes on each membrane. After filtration, EDTA
was added, and the solution was stored frozen in 15-ml aliquots
in acid-washed tubes (soaked in 10% HNOs overnight and rinsed at
least 10 times for each tube with milli Q water). BME was added before use.

The ATP eluate was collected in 10 fractions of 0.5 ml. The fractions (4–7) that corresponded to the 5-LO protein peak (see Fig.
1) were collected into carefully acid-washed Microfuge tubes. These
fractions were stored separately (not pooled) at −20 °C. An aliquot
was removed from each tube before freezing. Part of the aliquot was
used for the assay of enzyme activity, which covered the iron
concentration range of 0.05–500 ng/ml, and SDS-PAGE pattern. The remaining part of the aliquot was mixed with the same volume of 10 mg/ml bovine carbonic anhydrase (Sigma) and stored at −20 °C, for subsequent assay of enzyme activity.

Assay of 5-LO Activity—The reaction mixture contained 0.1 ml of
160 mM arachidonic acid, 5 mM 13-hydroperoxy-9,11-octadecadienoic
acid, 0.2 mM phosphatidylcholine, 50 mM Tris-Cl, pH 7.5, and various amounts of enzyme protein. After incubation at room temperature for 5 min, the reaction was stopped by adding 0.3 ml of MeCN/MeOH/HOAc (2:1:0.0027)
containing 1nmol of 17(S)-hydroxy-13,19(Z),15(E)-docosatrienoic
acid (17-OH-22:3). The precipitation was removed by centrifugation
and an aliquot of the supernatant was injected to a Nova-Pac C8
high performance liquid chromatography column (Waters) eluted with MeCN/MeOH/HOAc/HOAc (2:1:1:0.008) at 1.2 ml/min and monitored
at 234 nm. The amount of 5-HETE and 5-HPETE, which
were usually run in duplicates, and the data were mostly calculated using
a second order equation.

Iron Determination—Iron concentration was determined by graph-
ite furnace atomic absorption spectrophotometry using an instrument
(Perkin-Elmer 5000 Zeeman) equipped with an electrothermal atom-
ization unit (HGA-500), an automatic sample injector (AS-40), and a
computer (Perkin-Elmer 7500). A recorder (Perkin-Elmer 505) was
also connected for the visual analysis of nonspecific background sigs
and the compensated iron signal. The absorption at 248.3 nm (banc
pass 0.2 nm, low slit) from an iron hollow cathode lamp was measured
The electrothermal atomization unit was adjusted (with magnet on),
for maximum power heating and run with a program shown in Table 1.
This procedure gave a clear atomization signal without significant
carryover from the previous sample. Nevertheless, blanks (containing
0.015 M HNOs) were routinely inserted between all samples. As
calculated from these blanks, the detection limit of the graphite
furnace atomic absorption spectrophotometry was about 2 ng/ml
(detection limit (X) = Xnmol + SDDnmol). All measurements (standards or samples) were subtracted by the blank signal.

Twenty µl of sample (for preparation, see below) was injected onto a 1'Vov's platform in a pyrolytical graphite tube. The area under the
atomization signal peak was integrated by the instrument. Two
injections were carried out from each cup to give an average value of
the peak area. The instrument was zeroed before start against
an empty injection.

All utensils used in iron analysis were washed with 10% nitric acid.
Metal-free pipette tips (Bio-Rad) were rinsed 10 times with Milli Q
water before use. A number of tips were checked by holding 0.05 M
HNOs for 1 min, no iron was detected in the rinsing solution. Samples
and standards were diluted and prepared using a balance to achieve
good accuracy. Dilutions were made directly in the analysis cups. All
final samples contained 0.015 M HNOs to prevent the absorption of
trace metal onto the cup wall.

Iron standards were freshly prepared in 0.03 M HNOs by
dilution of a stock standard solution containing 1000 µg/ml of iron
in 1 M HNOs (British Drug House, United Kingdom). Five solutions
at concentrations of 10, 30, 50, 100, and 200 ng/ml were prepared.
Each of them was further diluted with the same amount of water in
analysis cups, for construction of an external standard curve. Since a
large number of samples were analyzed (100–150 cups in batch II, III,
and IV analyses), two to three sets of such standard solution
cups were prepared and analyzed at the beginning, the middle, and at
the end of each batch analysis to control any drifting of the instrument
conditions. Another iron standard (1000 µg/ml of iron in 0.1% HCl,
Aldrich) was compared with the one used routinely. When the two
standards were diluted in the same way, they gave practically identical
results.

The iron concentrations of the protein samples were determined
using either external or internal standards. The external standard
curve, which covered the iron concentration range of 0–100 ng/ml,
was linear up to about 50 ng/ml. The sample was diluted with water
and then mixed with an equal volume of 0.03 M HNOs, resulting in
iron concentrations between 30 and 60 ng/ml (in the analysis cup).
Total dilution was usually 4-fold for No. 4 fractions and 8-fold for
No. 5 fractions (from the AGATP column). These samples were
used for the iron determination, and the data were mostly calculated using
a second order equation.

About 40% of the samples was analyzed with internal standards
(standard additions). The protein sample was diluted with water and
then mixed with an equal amount of either of three standard solutions

| Step | Temperature | Ramp time | Hold time | Magnet and recorder start |
|------|-------------|-----------|-----------|--------------------------|
| Drying | 100 °C | 5 s | 25 s | 18 |
| Drying | 200 °C | 5 s | 10 s | 0 |
| Charring | 1300 °C | 6 s | 14 s | 18 |
| ATomization | 2550 °C | 6 s | 6 s | 0 |
| Cleaning | 2700 °C | 1 s | 1 s | 0 |
| Cooling | 200 °C | 1 s | 1 s | 0 |

*Integration of the peak area started 1 s after the start of this
step, with a total integration time of 8 s. The internal flow rate of
the purging gas (99.99% argon) was 50 ml/min, during this and the
following step.*
analyses showed that the slopes of the internal standard curves were within 10% variation of the slopes of the external standard curves, indicating that there was no significant interference from the sample matrix in the iron determinations.

The iron content of the proteins were calculated as the ratio of the iron and protein concentrations. The iron concentration was probably determined more accurately, because the iron standard and the iron in the samples were analyzed in the same final atomized form. For protein determination, we used the Bradford dye binding assay with bovine serum albumin as standard, which was found to be in agreement with amino acid analysis of 5-LO (12). The protein assay may have given errors around 10%.

The negative control sample contained a small amount of iron (about 2 ng/ml in the analysis cup). The iron concentration in the ATP buffer used in the last purification step was about the same as that in the negative control, suggesting that the contaminating E. coli proteins in the negative control did not contain significant amounts of iron. It was not possible to remove iron completely from the ATP buffer even after extensive filtration through metal chelating membranes. A negative control was included in each batch of samples that was purified for iron analysis. The iron and protein concentrations of the negative control were subtracted from the corresponding data of the other samples in the same batch, during the calculation of iron contents and specific activities (data in Tables III and IV corrected accordingly).

RESULTS

Purification—5-LO and mutated proteins were purified in two steps: ammonium sulfate precipitation and AGATP column chromatography (Table II). About 0.8 mg of 5-LO protein was obtained from 1 liter of E. coli expression culture. The whole process gave a 96-fold purification with 18% yield. The purity of the samples was greater than 95%, estimated by comparing the amount of protein in 5-LO samples with that in negative controls (see below).

The ammonium sulfate precipitation step was convenient for sample handling. 5-LO was not stable in the E. coli homogenate when kept at 4 °C for more than 1 day. However, the ammonium sulfate pellet could be stored at 4 °C for days, or at −20 °C for weeks, without significant loss of 5-LO activity.

The AGATP column was eluted with two solutions, one containing AMP and the other containing ATP. AMP elution removed an E. coli protein (about 48,000 daltons), whereas ATP eluted 5-LO (Fig. 1). AMP had some affinity to 5-LO, since more concentrated AMP solutions could elute the 5-LO protein. The condition of the column could affect the result. An old column reduced the yield of 5-LO protein (lost in AMP elution), whereas more of the 48,000-dalton contaminant appeared in the ATP eluate. The protein samples in Figs. 1 and 3 were obtained from a newly packed column and purified for iron analysis. The iron and protein concentrations of the negative control were subtracted from the corresponding data of the other samples in the same batch, during the calculation of iron contents and specific activities (data in Tables III and IV corrected accordingly).

| Step             | Total protein | Total activity | Yield | Specific activity | Purification |
|------------------|---------------|----------------|-------|------------------|--------------|
| Homogenate       | mg            | µmol          | %     | µmol/mg          | -fold       |
| (NH₄)₂SO₄        | 436.7         | 96.6          | 100   | 0.22             | 1           |
| AGATP column*    | 0.8           | 17.0          | 18    | 21.2             | 96          |

*Sup16 was used for the determination.

**TABLE II**

**Purification of nonmutated recombinant 5LO**

The starting material for the purification was 1 liter of E. coli expression culture (cell density, 3.85 optical density units). The data in this table correspond to the nonmutated 5LO sample in batch IV.

The samples in Fig. 2 were purified from a column which had been used for processing proteins from 40 to 50 liters of E. coli culture. After AGATP column chromatography, fractions 4–7 (protein concentrations 0.2–0.8 mg/ml) were collected and divided in three parts each. One was used for immediate analysis of enzyme activity, protein concentration, and for SDS-PAGE. Another part was mixed with an equal volume of 10 mg/ml bovine carbonic anhydrase and frozen quickly in dry ice/EtOH bath (for preservation of enzyme activity, see below). The third part was frozen without any addition, for subsequent iron analysis. Sometimes, when the samples for iron analysis were thawed, precipitates were found (see Table IV).

The negative control sample (purified from E. coli culture transformed with pTT73) contained 0.02–0.025 mg of protein (sum of fractions 4–7). Apparently similar amounts of the E. coli proteins were also found as contaminants in the purified samples of nonmutated 5-LO and all mutated proteins (Figs. 2 and 3). For most of the samples, the contaminants constituted 5% or less of the total protein. Some of the samples had
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To avoid depletion of substrate, the final concentration of the purified nonmutated 5-LO in the reaction mixture was 1–2 μg/ml. The activities of batch I and batch II samples are not included in Table IV, since these samples were assayed without preincubation. It was found later that too high enzyme concentrations in the assay lowered the apparent production of 5-HPETE and 5-HETE more than 10-fold, probably due to the exhaustion of substrate or to a relatively higher conversion to leukotriene A₄. For the same reason, the specific activities of 5-LO and its mutants, in the supernatants of cell homogenates, were underestimated in our previous report (20).

In order to detect possible low residual enzyme activity, the mutants were assayed with two protein concentrations. One was the same concentration as that of nonmutated 5-LO (for active mutants), and the other was 40–160 times higher (for inactive mutants).

Iron Contents and Specific Activities of 5-LO Mutants—The iron contents and specific activities of the 5-LO mutants divided these in three groups.

First, H372Q and H550Q contained basically no iron and they were also inactive (see Tables III and IV). The iron content of fraction 5 of H372Q in batch IV was from the analysis of the supernatant of a precipitated sample. When supernatants from other precipitated samples were analyzed, all of these gave higher iron content values than the corresponding nonprecipitated samples. This indicates that the denatured 5-LO protein released iron into solution. The iron content of the supernatant of the precipitated H372Q sample was not elevated, further proof that this protein was devoid of iron.

In the second group, H362Q, H390Q, and H399Q contained about the same amount of iron as nonmutated 5-LO, whereas the enzyme activities were reduced. The recovery of enzyme activity of H362Q in AGATP column purification was very low (1–2%) when compared with nonmutated 5-LO (20%). This was apparently related to the instability of this mutated protein, since the yield of protein was similar to that of nonmutated 5-LO. Carbonic anhydrase could revert this inactivation to some extent. Thus, after storage at −20 °C (in presence of carbonic anhydrase), the activity of some samples of purified H362Q increased about 5-fold (from 0.5 to about 2.5 μmol/mg). However, if the yield of enzyme activity in the AGATP chromatography had been the same for H362Q as for normal 5-LO, the specific activity of this mutated protein should have been 5–10 μmol/mg. Apparently, freezing and thawing in the presence of the carbonic anhydrase only rescued part of the activity. Possibly, this treatment could only revert an ongoing or very recent inactivation of the H362Q protein. On the other hand, carbonic anhydrase could not reactivate an inactivated nonmutated 5-LO.

H390Q, and to some extent H399Q, were obtained in very low amounts after purification. These preparations thus had higher percentages of contaminating proteins. In the iron concentration analysis, H390Q gave only a 2–3-fold higher signal peak area than the negative control. Therefore, the iron content data regarding H390Q are only approximate.

In our previous study (20), a temperature-sensitive character of H390Q and H399Q was observed. E. coli cultures at 37 °C gave very low enzyme activities for these two mutants. In this study, these mutants were expressed at 27 °C (batch II and IV) and 18 °C (batch III). There was no dramatic difference in specific activity between the samples. It thus appears that at certain temperature (between 27 and 37 °C), the proper folding of these mutated proteins was significantly affected.
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The iron contents were calculated after corrections of the iron and protein concentrations, by subtractions of the corresponding data for the negative control sample in the same batch (see "Results"). Negative values are listed as zero. In some batches, a protein was prepared two or three times (a, b, c). ppt means that precipitates were found when the samples were thawed (see "Results").

### TABLE III
Iron contents (mol/mol) of 5LO and mutated proteins

| Batch | 5LO | H362Q | H367Q | H367N | H367S | H372Q | H390Q | H399Q | H550Q |
|-------|-----|-------|-------|-------|-------|-------|-------|-------|-------|
| Ia    |     |       |       |       |       |       |       |       |       |
| 4     | 0.84|       |       |       |       |       |       |       |       |
| 5     | 0.84|       |       |       |       |       |       |       |       |
| Ib    |     |       |       |       |       |       |       |       |       |
| 4     | 0.74|       |       |       |       |       |       |       |       |
| 5     | 0.75|       |       |       |       |       |       |       |       |
| II    |     |       |       |       |       |       |       |       |       |
| 4     | 1.15| 1.21  | 0.47  | 0.04  | 1.98  | 1.20  | 0     |       |       |
| 5     | 0.76| 1.01  | 0.43  | 0.07  | 0.72  | 0.78  | 0     |       |       |
| IIIa  |     |       |       |       |       |       |       |       |       |
| 4     | 1.69| 0.72  | 0.58  | 0.01  | 1.01  | 0.79  | 0.02  |       |       |
| 5     | 0.91| 0.85  | 0.58  | 0.01  | 0.60  | 0.71  | 0.02  |       |       |
| IIIb  |     |       |       |       |       |       |       |       |       |
| 4     |     |       |       |       |       |       |       |       |       |
| 5     |     |       |       |       |       |       |       |       |       |
| IIIc  |     |       |       |       |       |       |       |       |       |
| 4     |     |       |       |       |       |       |       |       |       |
| 5     |     |       |       |       |       |       |       |       |       |
| IV    |     |       |       |       |       |       |       |       |       |
| 4     | 0.89| 0.66  | ppt   | 0     | 0.71  | 1.04  | 0.01  |       |       |
| 5     |     | 0.70  | ppt   | 0     | 0.76  | ppt   | 0.04  |       |       |
| 6     |     |       |       |       |       |       |       |       |       |
| Va    |     |       |       |       |       |       |       |       |       |
| 4     | 0.79|       |       |       |       |       |       |       |       |
| 5     | 0.90|       |       |       |       |       |       |       |       |
| V     |     |       |       |       |       |       |       |       |       |
| 4     |     |       |       |       |       |       |       |       |       |
| 5     |     |       |       |       |       |       |       |       |       |
| Mean  | 0.86| 0.90  | 0.52  | 0.17  | 0.48  | 0.03  | 0.81  | 0.89  | 0.02  |

*Numbers indicate fraction number.

### TABLE IV
Specific activities (μmol/mg) of 5LO and mutated proteins

Activity was defined as the production of 5-HETE and 5-HPETE with less than 25% consumption of substrate, during a 5-min incubation at room temperature. The activities for samples from batches III and IV were determined for aliquots that had been stored frozen in the presence of carbonic anhydrase, to prevent loss of activity (see "Materials and Methods"). The data for samples in batch V were from assays performed immediately after the AGATP column chromatographies. In some batches, a protein was prepared two or three times (a, b, c).

| Batch | 5LO | H362Q | H367Q | H367N | H367S | H372Q | H390Q | H399Q | H550Q |
|-------|-----|-------|-------|-------|-------|-------|-------|-------|-------|
| IIIa  |     |       |       |       |       |       |       |       |       |
| 4     | 28.5| 2.5   | 0     | 0     | 11.2  | 10.0  | 0     |       |       |
| 5     | 28.4| 3.1   | 0     | 0     | 8.8   | 8.5   | 0     |       |       |
| IIIb  |     |       |       |       |       |       |       |       |       |
| 4     |     |       | 0.6   | 0     |       |       |       |       |       |
| 5     |     |       | 0.7   | 0     |       |       |       |       |       |
| IIIc  |     |       | 2.5   | 0     |       |       |       |       |       |
| 4     |     |       | 2.7   | 0     |       |       |       |       |       |
| 5     |     |       |       |       |       |       |       |       |       |
| IV    |     |       |       |       |       |       |       |       |       |
| 4     | 23.9| 1.9   | 0     | 0     | 13.9  | 14.1  | 0     |       |       |
| 5     | 20.9| 2.4   | 0     | 0     | 16.1  | 15.8  | 0     |       |       |
| 6     | 20.8|       |       |       |       |       |       |       | 11.4  |
| Va    |     |       |       |       |       |       |       |       |       |
| 4     | 20.8|       |       |       | 0     | 0     |       |       |       |
| 5     | 21.0|       |       |       | 0     | 0     |       |       |       |
| Vb    |     |       |       |       |       |       |       |       |       |
| 4     |     |       |       |       | 0     | 0     |       |       |       |
| 5     |     |       |       |       | 0     | 0     |       |       |       |
| Mean  | 23.5| 2.1   | 0     | 0     | 0     | 12.5  | 12.0  | 0     |

In the third group of mutated proteins, H367Q, H367N, and H367S had about 0.5, 0.2, and 0.5 mol of iron/mol of protein, respectively. They were all inactive. These mutated proteins were consistently obtained in good amounts after purification, some gave higher yields than nonmutated 5-LO. However, H367S appeared more sensitive to degradation than the other mutated proteins in this study (Fig. 3). The four prominent degradation bands in the SDS-PAGE corre-
The purpose of this study was to identify possible iron ligands in human 5-LO by site-directed mutagenesis. Considerable effort was also spent on the development of a simple, fast, and reproducible procedure for enzyme purification. The fast procedure was not only required because of the large number of protein samples needed to be purified for iron content analysis, but also because of the unstable character of the human 5-LO which has been described to be susceptible to oxygenation (12, 28). Two purification procedures have been reported for human 5-LO (28–31). The ATP column chromatography method is faster and simpler. However, from the previous reports (29–31), it seemed that a reasonable purity could only be achieved by this method when 5-LO had been substantially enriched in the starting material. The recombinant human 5-LO used in this study was expressed in E. coli to a relatively low concentration, it did not exhibit a distinguishable band when the whole cell homogenate was analyzed on SDS-PAGE. To purify 5-LO in this starting material by ATP column chromatography, two measures were taken. First, strong washing cycles were integrated into the sample loading procedure, to avoid the nonspecific binding of other proteins to the ATP ligand on the column. Second, an AMP solution was used in the initial elution step to remove a specifically bound E. coli protein from the column, before the final elution of 5-LO. With this procedure, the enzyme was obtained with a purity that was satisfactory for the purpose of this study.

During the study of the iron content, six nonmutated 5-LO samples, were prepared to serve as controls or for trials of the procedure. From the analyses of these samples, it was shown that 5-LO contained 0.86 mol of iron/mol of enzyme protein with a range of 0.74–1.15. This is in agreement with a previous study, in which 5-LO was also concluded to contain 1 mol of iron/mol of protein (12). In contrast, however, we did not encounter substantial loss of iron in these samples. Possibly, this is due to different procedures used in the sample handling, or more likely, to the different cells used for expression of recombinant human 5-LO (insect cells versus E. coli). The recombinant 5-LO may have been modified differently in the two systems.

The association of iron to soybean lipoxygenase-1 has been suggested to involve 3–5 histidine residues, from a study based on extended x-ray absorption fine structure analysis (18). Interestingly, when the cDNA sequences of various lipoxygenases became available, 6 histidine residues were found to be conserved in all cloned enzymes. In this study, the 6 histidine residues in 5-LO were replaced by glutamine individually, and the iron contents of the mutated proteins were analyzed. The following observations were made. 1) The changes of His72 and His500 yielded proteins no longer capable of binding iron. These mutants were also inactive. 2) The replacements of His362, His390, and His399 did not affect the iron content of the enzyme, but gave lower enzyme activity. 3) Substitution of His367 gave a protein which had a partial iron content, 0.5 mol/mol (iron/protein). When this residue was replaced by two other amino acids, asparagine or serine, the iron contents were still partial, 0.2 and 0.5 mol/mol respectively. None of the His367 mutants had enzyme activity.

The absence of iron in the mutants of His372 and His500 indicates that these 2 histidines are likely to be iron ligands in the enzyme. However, the possibility that the substitutions lead to a general conformational change cannot be completely excluded. Such a change could block the iron binding even though the iron ligands still exist. Nevertheless, by choosing glutamine as the substitute for histidine, we assume that this possibility is small. The glutamine-histidine exchange has been shown to have the highest evolutionarily conservative replacement score according to the mutation data matrix (32).

In contrast to the His372 and His500 mutants, the replacements of His362, His390, and His399 did not significantly affect the iron content of the enzyme. This indicates that these histidine residues are not likely to be involved in the iron binding. The importance of these conserved residues may instead be related to the maintenance of the stability of the enzyme or to facilitate proper protein folding during the enzyme synthesis. In line with this, striking instability or significantly lower expression levels were encountered, when these amino acids were substituted. These mutants have also been studied in an insect cell expression system (25). It would be valuable to compare the stability of the mutated proteins expressed in prokaryotic versus eucaryotic cells.

Perhaps the most interesting mutants in this study were H367Q, H367N, and H367S. His367 is located in a part of the 5-LO sequence (367–381) which has homology to the interface binding domain of human lipoprotein lipase and rat hepatic lipase (33). Replacements of 3 conserved amino acids (His367, His372, Glu386) in this region have all abolished the enzyme activity completely (20, 20–24). It is possible that not only the 3 amino acids, but also the region, is important for the enzyme activity.

Two explanations for the partial iron content of the His367 mutants seem plausible. One is that His367 is not an iron ligand, but is located at the active site, possibly of importance for substrate binding. The active site should be very close to the iron binding site, possibly including some of the iron ligands. Thus, substitution of His367 could interfere with the iron binding because of close proximity to some of the ligands, for example His372. The other explanation is that His367 may be one of the iron ligands, but less important. Without this ligand, the iron could still be bound to the enzyme by the remaining ligands, but less tightly. This improper binding could have shifted the iron from its proper location so that it is no longer able to accept or donate electrons, from or to the reaction intermediates. For soybean lipoxygenase, it has been proposed that an exogenous ligand (tentatively a reaction intermediate) would replace one of the iron ligands of the protein, during the catalytic cycle (34). Assuming that this is valid also for human 5-LO, and because of the peculiar properties of the His367 mutants, it is tempting to speculate that His367 could be a replaceable ligand.

The final answer of which amino acids that bind to iron in 5-LO will be obtained when the three-dimensional structures have been solved. Comparisons with the results from mutagenesis experiments will contribute to the understanding of the relevance of the various ligands for the binding and function of iron in lipoxygenase catalysis.

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