Mutation analysis of the human 5-lipoxygenase C-terminus: Support for a stabilizing C-terminal loop

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Abstract

Lipoxygenases contain prosthetic iron, in human 5-lipoxygenase (5LO) the C-terminal isoleucine carboxylate constitutes one of five identified ligands. ATP is one of several factors determining 5LO activity. We compared properties of a series of 5LO C-terminal deletion mutants (one to six amino acid residues deleted). All mutants were enzymatically inactive (expected due to loss of iron), but expression yield (in *E. coli*) and affinity to ATP–agarose was markedly different. Deletion of up to four C-terminal residues was compatible with good expression and retained affinity to the ATP-column, as for wild-type 5LO. However when also the fifth residue was deleted (Asn-669) expression yield decreased and the affinity to ATP was markedly diminished. This was interpreted as a result of deranged structure and stability, due to loss of a hydrogen bond between Asn-669 and His-399. Mutagenesis of these residues supported this conclusion. In the structure of soybean lipoxygenase-1, a C-terminal loop was pointed out as important for correct orientation of the C-terminus. Accordingly, a hydrogen bond appears to stabilize such a C-terminal loop also in 5LO.
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1. Introduction

5-Lipoxygenase (5LO) catalyzes the two initial steps in leukotriene biosynthesis, converting arachidonic acid to 5(S)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE) and subsequently into the epoxide leukotriene A$_4$ [1]. The leukotrienes are inflammatory mediators; several findings now imply a role for 5LO and leukotrienes in atherosclerosis, see Refs. [2,3] for reviews, and recently in development of aortic aneurysm [4]. Other recent connections to pathophysiology concern pulmonary hyper-tension [5], multiple organ failure [6] and ischemia–reperfusion injury [7]. 5LO is also discussed in relation to cancer [8].

A model of the 5LO structure, based on the crystal structure of the ferrous form of rabbit reticulocyte 15LO [9], consists of an N-terminal β-barrel (residues 1–114) and a larger C-terminal catalytic domain (residues 121–673); compare Fig. 5. Ca^{2+} binds to the C2-like β-barrel [10–12] leading to association with the nuclear membrane [12,13] and thus increased enzyme activity. MAP kinases phosphorylate 5LO on Ser-271 and Ser-663 (in the catalytic domain) and p38 MAPK pathways can activate 5LO during cell stress [14–17]. On the other hand, phosphorylation on Ser-523 by protein kinase A inhibited 5LO activity [18]. ATP is another factor stimulating 5LO activity, which also binds to 5LO [19,20], but the ATP binding site remains undefined. The large catalytic domain contains the iron,

which is required for catalysis. Four amino acid side chains constitute iron ligands (His-367, His-372, His-550, and Asn-554), while one of the oxygens in the carboxylate group of the C-terminal Ile-673 is the fifth ligand. Of the five ligands, His-372, His-550, and the C-terminal Ile appear to constitute a 2-His-1-carboxylate facial triad [21]. These are necessary to anchor the prosthetic iron; for review, see Ref. [22].

The first lipoxygenase to be crystallized was soybean lipoxygenase-1 (SLO-1) [23,24] and mammalian lipoxygenases (including 5LO) have been modelled also on the basis of this structure [25]. It was thus suggested that mammalian lipoxygenases, as SLO-1, should contain a C-terminal loop important for the geometry of the C-terminal iron ligand. Here we report mutagenesis results which support the presence of a hydrogen bond connecting His-399 with Asn-669, leading to a C-terminal loop in human 5LO. In this study, also Trp-75 and Trp-201, previously found to react with ATP analogues [20] were subjected to mutagenesis.

2. Materials and methods

2.1. Reagents

Restriction enzymes, T7 sequencing kit, and other molecular biology reagents were from Amersham Pharmacia. Oligonucleotides were purchased from Scandinavian Gene Synthesis (Köping, Sweden) or from Pharmacia. Solvents for HPLC were from Rathburn Chemicals (Walkerburn, Scotland). Other chemicals were from Sigma.

2.2. Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis was performed using the selection principle described by Kunkel using the Muta-Gene kit from Bio-Rad. The plasmid pT3-5LO [26] was transformed into the Dut^- Ung^- *E. coli* strain CJ236 and single-strand uracil containing pT3-5LO cDNA was extracted after infection with the helper phage M13K07. Mutated cDNA was transformed into the Dut^+ Ung^+*E. coli* strain MV1190. Plasmid DNA was extracted from several clones using a Magic Miniprep kit (Promega, Madison, WI). Mutated clones were identified by sequencing. See Table 1 for list of mutants.

2.3. Expression in E. coli and extraction of protein

E. coli MV 1190 harbouring mutated plasmid was grown in modified M9 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; $FeSO_4$, 5 μM; glycerol, 2% (w/v); and casein hydrolysate, 2 mg/ml) containing ampicillin, 150 μg/ml. A suitable volume (0.1 or 1 l) was inoculated with overnight culture (0.5 or 10 ml, in LB) and incubated at 18 or 27 °C. When cells had grown to an OD of circa 0.2 (620 nm), isopropyl β-D-

Table 1
5LO mutants included in this study

Mutant	Codon change
C-2	Nucleotide 2017–2022 deleted
C-3	Nucleotide 2014–2022 deleted
C-4	Nucleotide 2011–2022 deleted
C-5	Nucleotide 2008–2022 deleted
C-6	Nucleotide 2005–2022 deleted
H399A	CAC→GCC
H399G	CAC→GGC
P668E	CCG→GAG
N669A	AAC→GCC
W75R	TGG→AGG
W75F	TGG→TTC
W75S	TGG→TCG
W75A	TGG→GCG
W201R	TGG→AGG
W201F	TGG→TTC
W201S	TGG→TCG
W201A	TGG→GCG

thiogalactopyranoside (IPTG) was added to a concentration of 0.2 mM. At OD circa 0.7 (620 nm), the cells were harvested by centrifugation at 10,000 ×g for 10 min, the cell pellet was snap-frozen and kept at −20 °C.

For extraction, frozen pellets were suspended in 10/50 ml of sonication buffer (for 0.1/1 l cultures) and incubated for 5 min at room temperature followed by 25 min on ice. The sonication buffer contained 50 mM Triethanolamine–HCl, pH 8.0, 5 mM EDTA, 2 mM dithiothreitol, 60 μg/ml soybean trypsin inhibitor, 500 μg/ml lysozyme, and 1 mM phenylmethanesulfonylfluoride. The cell suspension (cooled in ice-water) was sonicated with a MSE MK2 150 W ultrasonic disintegrator (3 × 15 s, amplitude 18). The resulting homogenate was centrifuged at 15,000 ×g for 15 min, giving supernatant S15. For 1 l cultures, the yield was increased when the 15,000 ×g pellet was subjected to repeated sonication (after resuspension in 25 ml of sonication buffer). Thus, for 1 l cultures sup 15 from two centrifugations were pooled (total 75 ml).

2.4. Ammonium sulfate precipitation

Proteins in S15 (10 or 75 ml, see above) were precipitated by ammonium sulfate. To the cold sample, a saturated solution of $(NH_4)_2SO_4$ was added, to reach 60% (v/v). After stirring on ice for 40 min, the precipitate was collected by centrifugation at 15,000 ×g for 30 min, snap-frozen, and stored at −20 °C. Just prior to chromatography on agarose–ATP, the ammonium sulfate pellet was resuspended in 7 ml (for 100 ml culture) or 25 ml (for 1 l culture) of buffer A (see below) and centrifuged at 100,000 ×g for 60 min, giving supernatant S100.

2.5. Chromatography on ATP–agarose

For agarose–ATP affinity chromatography of samples from 100 ml cultures, a column (0.5 × 5, 1 ml) was packed