Analysis of a nucleotide-binding site of 5-lipoxygenase by affinity labelling: binding characteristics and amino acid sequences

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5-Lipoxygenase (5LO) catalyses the first two steps in the biosynthesis of leukotrienes, which are inflammatory mediators derived from arachidonic acid. 5LO activity is stimulated by ATP; however, a consensus ATP-binding site or nucleotide-binding site has not been found in its protein sequence. In the present study, affinity and photoaffinity labelling of 5LO with 5'-*p*-fluorosulphonylbenzoyl-adenosine (FSBA) and 2-azido-ATP showed that 5LO bound to the ATP analogues quantitatively and specifically and that the incorporation of either analogue inhibited ATP stimulation of 5LO activity. The stoichiometry of the labelling was 1.4 mol of FSBA/mol of 5LO (of which ATP competed with 1 mol/mol) or 0.94 mol of 2-azido-ATP/mol of 5LO (of which ATP competed with 0.77 mol/mol). Labelling with FSBA prevented further labelling with 2-azido-ATP, indicating that the same binding site was occupied by both analogues. Other nucleotides (ADP, AMP, GTP, CTP and UTP) also competed with 2-azido-ATP labelling, suggesting that the site

was a general nucleotide-binding site rather than a strict ATP-binding site. Ca²⁺, which also stimulates 5LO activity, had no effect on the labelling of the nucleotide-binding site. Digestion with trypsin and peptide sequencing showed that two fragments of 5LO were labelled by 2-azido-ATP. These fragments correspond to residues 73–83 (KYWLNDWDWYLK, in single-letter amino acid code) and 193–209 (FMHMFQSSWNDFADFEK) in the 5LO sequence. Trp-75 and Trp-201 in these peptides were modified by the labelling, suggesting that they were immediately adjacent to the C-2 position of the adenine ring of ATP. Given the stoichiometry of the labelling, the two peptide sequences of 5LO were probably near each other in the enzyme's tertiary structure, composing or surrounding the ATP-binding site of 5LO.

Key words: ATP-binding site, azido-ATP, fluorosulphonylbenzoyl-adenosine.

INTRODUCTION

5-Lipoxygenase (5LO) catalyses the first two reactions in the leukotriene biosynthesis pathway, which convert arachidonic acid into 5-hydroperoxyeicosatetraenoic acid and then to leukotrienes A₄, B₄, C₄, D₄ and E₄. Leukotriene B₄ is a potent chemotactic agent, which attracts leukocytes to the site of inflammation. Leukotrienes C₄, D₄ and E₄ induce the constriction of bronchial and vascular smooth muscle. They have been implicated as mediators in asthma and other types of allergic reaction [1]. Recent reports have also shown that 5LO is involved in tumour cell proliferation [2–7] and is related to primary pulmonary hypertension [8–12].

The activity of 5LO is stimulated by ATP, and to a smaller extent by other nucleotides such as ADP, AMP, CTP, UTP and cAMP [13]. The magnitude of the stimulation is in the range of 2–6-fold; 5LOs from various sources all exhibit the same property [14–18]. One report indicated that ATP is not hydrolysed during the stimulation, and that adenosine 5'-[γ-thio]triphosphate stimulates 5LO better than ATP [19]. Several studies have shown that the ATP-stimulation requires Ca²⁺ [13,14,20,21], although a recent report demonstrated that Ca²⁺ is not necessary for the stimulation of purified human 5LO [22]. Because 5LO co-precipitates with ATP [19] and can be purified by ATP-affinity column chromatography [23–26], the stimulation of 5LO by ATP is thought to be via a direct interaction of the two molecules.

Interestingly, 5LO does not contain consensus ATP-binding or nucleotide-binding sites. The protein sequences of four cloned 5LOs [27–31] have been analysed with a database for protein sites, patterns and profiles (Prosite; Swiss Institute of Bioinformatics). None of them yet contains an ATP-binding site or a nucleotide-binding site registered in the databank. In addition, no nucleic acid (DNA or RNA)-binding site was found in the 5LO sequences. Thus the site that 5LO uses for binding ATP might be an unconventional site.

For these reasons we examined the binding characteristics of the ATP-binding site of 5LO and the amino acid sequences involved in the binding. We used affinity and photoaffinity labelling with two types of ATP analogue, 5'-*p*-fluorosulphonylbenzoyl-adenosine (FSBA) [32,33] and azido-ATP [34–36].

EXPERIMENTAL

Materials

FSBA, tosylphenylalanylchloromethane [Tos-Phe-CH₂Cl ('TPCK')]-labelled-trypsin, ATP-agarose (catalogue no. A2767) and iminodiacetic acid-immobilized Sepharose 6B fast flow were obtained from Sigma (St Louis, MO, U.S.A.). 5'-*p*-[adenine-8-¹⁴C]FSBA was purchased from New England Nuclear (Boston, MA, U.S.A.). 2-Azido-ATP, 8-azido-ATP, 2-azido-[γ-³²P]ATP and 8-azido-[γ-³²P]ATP were obtained from Research Product

Abbreviations used: 5LO, 5-lipoxygenase; FSBA, 5'-*p*-fluorosulphonylbenzoyl-adenosine; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK').

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International Corp. (Mt Prospect, IL, U.S.A.) and from Affinity Labelling Technologies (Lexington, KY, U.S.A.).

Preparation of 5LO

Escherichia coli strain MV1190 or DG1 [37] was transformed with an expression vector, pT3-5LO, which contains a human 5LO cDNA insert [38]. An overnight culture was grown at 30 °C in Luria–Bertani medium containing 150 µg/ml ampicillin. In a fermenter, 20 litres of super-rich medium containing 20 g/l tryptone, 20 g/l yeast, 20 g/l casamino acids (casein hydrolysates), 10 g/l meat extract and 100 µg/ml ampicillin was inoculated with 200 ml of overnight culture and incubated at 30 °C. Protein expression was induced after 6–7 h (D_{620} 1.0–1.5) by the addition of isopropyl β -D-thiogalactoside to a final concentration of 100 µM. After a further 12–14 h, the cells were harvested by continuous-flow centrifugation. The cell pellet (approx. 130 g) was snap-frozen and kept at –70 °C. The frozen cells were disrupted by compression (X-pression) and stored at –70 °C until use.

The *E. coli* strain DG1 is devoid of glycerol kinase, which constitutes a minor contaminant in the first few 5LO fractions collected from the ATP-agarose column. 5LO used in some of the experiments in this study was expressed in DG1, but because the DG1-expressed 5LO exhibited lower specific activity, MV1190-expressed 5LO was used in most of the experiments.

Approximately 40 g of X-pressed cells was thawed and homogenized in a juice blender (Atomix; MSE, Crawley, Surrey, U.K.) in 250 ml of buffer containing 50 mM triethanolamine/HCl (pH 8.0), 5 mM EDTA, 2 mM dithiothreitol, 60 µg/ml soybean trypsin inhibitor and 1 mM PMSF. The suspension was centrifuged at 20000 *g* for 40 min. The supernatant was collected and kept on ice; the pellet was resuspended in 50 ml of the above buffer, sonicated four times for 15 s with an MSE Mk2 150 W ultrasonic disintegrator and centrifuged at 20000 *g* for 50 min.

Supernatants from the two centrifugations were pooled, mixed with 100 ml of Sepharose Q (Pharmacia) and stirred at 4 °C for 40 min. The protein-bound Sepharose beads were washed with 250 ml of buffer A [50 mM triethanolamine/HCl (pH 7.3)/2 mM EDTA/10 mM 2-mercaptoethanol], followed by 100 ml of 0.1 M NaCl in buffer A. Bound proteins, including 5LO, were desorbed from the beads batchwise in four 100 ml volumes of 0.6 M NaCl in buffer A, followed by 100 ml of 0.8 M NaCl in buffer A. The supernatants were combined (500 ml) and satd ($(\text{NH}_4)_2\text{SO}_4$) was added to a final concentration of 60%–satd. After stirring of the supernatants on ice for 40 min, the precipitate was collected by centrifugation at 16000 *g* for 35 min, then snap-frozen and stored at –20 °C.

An $(\text{NH}_4)_2\text{SO}_4$ -precipitated pellet from approx. 750 ml of 60%–satd $(\text{NH}_4)_2\text{SO}_4$ suspension was resuspended in 30 ml of buffer A and centrifuged at 100000 *g* for 1 h. The supernatant (sup100) was loaded on an FPLC column packed with 8 ml of ATP-agarose at a flow rate of 1.0 ml/min. After every 10 ml of loading, a 10 ml wash was performed by passing through 2.5 ml of buffer A, 2.5 ml of linear gradient up to 100% buffer B (1 M NaCl in buffer A), 2.5 ml of gradient back to 100% buffer A, and finally 2.5 ml of buffer A. A final last step with 5 ml of 100% buffer B and 5 ml of 100% buffer A completed the loading and washing process. Bound proteins were eluted with 12 mM AMP (15 ml, in buffer A containing 100 mM NaCl) at a flow rate of 0.8 ml/min, followed by 12 mM ATP (25 ml, in buffer A containing 100 mM NaCl) at 0.4 ml/min. The elution fractions were collected at 4 min per tube. To the ATP eluate fractions (T3–T9, 1.6 ml in each) were added 200 µl of 100% glycerol, 3.6 µl of

stabilizing proteins (glutathione peroxidase and superoxide dismutase, to concentrations of 0.1 unit/ml and 1 µg/ml respectively, [39]) and 19 µl of 1 M ATP (to T3–T5 only). The samples were snap-frozen and stored at –70 °C. Transportation of the samples across countries was made at ambient (non-frozen) temperature for 2 days. In these cases, samples were frozen after the shipment, not before, and vials were sealed with the solution filled to the brim. No significant loss of activity was observed under transport conditions.

ATP-free 5LO was prepared immediately before the labelling experiments by one of the following two methods: (1) desalting on PD-10 columns followed by anion-exchange chromatography; or (2) desalting on a Fast Desalting FPLC column. The first method was used for the FSBA experiments; the second was used for the azido-ATP experiments. In the first method, a 1.5 ml 5LO solution was passed consecutively through two PD-10 columns (Pharmacia) equilibrated with buffer MQ1 [50 mM Tris/HCl (pH 7.5)/50 mM NaCl/1 mM EDTA]. The eluate was loaded on a 1 ml MonoQ column (Pharmacia) equilibrated with the same buffer. With a linear gradient of 0.05–1 M NaCl at a flow rate of 1 ml/min, 5LO was eluted at 0.20–0.22 M NaCl.

In the second method, 0.5 ml of 5LO solution was loaded on a Fast Desalting column (Pharmacia) equilibrated with 50 mM Tris/HCl (pH 7.0)/50 mM NaCl/0.1 mM EDTA at a flow rate of 0.5 ml/min. Fraction collection was started 3 min after injection, at 0.25 ml per tube. 5LO was eluted in fractions 5–7.

Protein assay

Protein concentration was determined as described by Bradford [40] with a protein assay kit from Bio-Rad; BSA was used as a standard.

FSBA labelling

Non-radioactive FSBA was used for the determination of the labelling time course and dose-dependence. The labelling solution (100 µl) contained buffer MQ1, FSBA (0–1 mM, added in 2 µl of DMSO) and 5LO (30–50 µg). Incubation was performed at 30 °C for 0–120 min. Aliquots of the labelling mixture were diluted 50-fold or 100-fold in the assay mixture and analysed for 5LO activity.

To estimate the stoichiometry of FSBA incorporation, a stock solution of radioactive FSBA was prepared by the evaporation of 460 µl of 5'-*p*-[adenine-8- ^{14}C]FSBA [NEN; 48 mCi/mmol, 0.42 mM in 95% (v/v) ethanol] under N_2 , followed by dissolution in 46 µl of 50 mM non-radioactive FSBA in DMSO, yielding a final concentration of 54.2 mM FSBA and a specific radioactivity of 3.8 mCi/mmol. MonoQ-purified 5LO (10–20 µM) and 5'-*p*-[adenine-8- ^{14}C]FSBA (1 mM) were incubated in buffer MQ1 in the absence or the presence of ATP (50 mM) in a total volume of 1 ml for 2 h at 30 °C. The reaction was terminated by the addition of 50 mM 2-mercaptoethanol. To liberate non-covalently bound FSBA from 5LO, 0.63 g of guanidinium chloride was added to the reaction solution and incubated for 20 min at 60 °C. The excess of reagents was removed by gel filtration at room temperature on a Sephadex G-25 column (1.6 cm $\times$ 25 cm) (Pharmacia) equilibrated with 50 mM Tris/HCl, pH 7.5, containing 5 M guanidinium chloride. The column was developed at a flow rate of 1.5 ml/min and 1 ml fractions were collected while UV absorbance was monitored at 280 nm. The protein concentration and radioactivity of the fractions of interest were measured.

Labelling with 2-azido-ATP or 8-azido-ATP

Immediately before labelling, 2-azido-ATP and 2-azido- $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$, which were both in a triethylammonium salt form in methanol, were mixed and dried under vacuum. Tris/HCl buffer (10 mM, pH 7.0) was added and a stock solution of 250 μM containing $(0.5\text{--}1) \times 10^6$ c.p.m./ μl was made. An aliquot of the stock solution was then added to a labelling solution (final volume 25 μl) containing 10 mM Tris/HCl, pH 7.0, and 1.5–2.5 μg of 5LO. In competition experiments, various amounts of competitor were also present. Labelling involved first incubating the solution on ice for 2–5 min, then irradiating it with UV (254 nm) for 10–120 s at a distance of 5 cm from the solution. SDS/PAGE loading buffer was then added and the samples (without being boiled) were separated on SDS/PAGE with a Phast system (Pharmacia) with a 6/4 comb, 10–15% (w/v) gradient gel and SDS buffer strips. The gels were stained with Coomassie Brilliant Blue for 15 min, destained for 10 min and dried with hot air for 10 min. 5LO bands and free-ligand bands were excised and counted for radioactivity; a segment of gel between the bands was counted as background. The procedure for large-scale labelling is described in the section on peptide preparation and sequencing. The labelling procedure was identical for both 2-azido-ATP and 8-azido-ATP, except that 2-fold the specific radioactivity was used in the 8-azido-ATP labelling.

Activity assay

In the FSBA labelling studies, the assay mixture contained 100 mM Tris/HCl (pH 7.5), 1.2 mM EDTA, 2 mM CaCl_2 , 100 μM arachidonic acid, 10 μM 13-hydroperoxy-9,11-octadecadienoic acid, 20 $\mu\text{g}/\text{ml}$ egg-yolk phosphatidylcholine and an aliquot of the labelling solution. In the azido-ATP labelling study, Ca^{2+} and 13-hydroperoxy-9,11-octadecadienoic acid were omitted from the assay mixture. The 5LO reaction was performed at room temperature for 5 min; products were separated by HPLC with a Nova-Pak C_{18} column (Waters). An isocratic solvent system, containing acetonitrile/water/acetic acid (60:40:0.2, by vol.) at 1.2 ml/min flow rate, was used for the separation. 5LO activity was calculated as the sum of the production of 5-hydroxy-eicosatetraenoic acid and 5-hydroperoxyeicosatetraenoic acid, the amounts of which were estimated with an internal standard, 17-hydroxydocosatetraenoic acid, added to the assay mixture at the end of the incubation.

Peptide separation and sequencing

5LO (1–1.5 mg; 13–19 nmol) was mixed with 100–150 nmol of 2-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ $[(1\text{--}4) \times 10^7$ c.p.m.] in a total volume of 2–3 ml. The mixture was incubated on ice for 5 min and transferred to a small weighing boat in three batches, each irradiated at 254 nm for 30 s. Ice-cold trichloroacetic acid was added to the labelling solution to a final concentration of 10% (w/v) and the protein was precipitated on ice for 30 min. The precipitated 5LO was collected by centrifugation at 15000 g for 15 min at 4 °C, and washed twice with ice-cold methanol.

The protein pellet was dissolved in 0.6 ml of 8 M urea and 0.12 ml of 29% (v/v) NH_4OH , neutralized with 0.06 ml of acetic acid and diluted in 2.1 ml of 0.1 M ammonium acetate, pH 8.0. Tos-Phe- CH_2Cl -trypsin was added at a trypsin-to-5LO ratio of 1:20 (w/w). Digestion was performed at 37 °C for 12–16 h; the completion of the digestion was confirmed by SDS/PAGE and autoradiography.

The digest was diluted 15-fold in buffer M1 (100 mM ammonium acetate, pH 5.9) and loaded on a 1.4 ml Al^{3+} -charged metal-chelating column (iminodiacetic acid-immobilized Sepha-

rose 6B fast flow). The Al^{3+} -charging was performed by passing 10 ml of 50 mM AlCl_3 , 15 ml of water and 10 ml of buffer M1 over the column. After being loaded, the column was washed with 15 ml of buffer M1, 5 ml of buffer M2 (0.5 M NaCl in buffer M1), followed by 5 ml of buffer M1. Elution was performed by alternately applying 0.8 ml of 20 mM K_2HPO_4 in 100 mM ammonium acetate, pH 8.0, and stopping column flow for 10 min. Fractions were collected in 5 ml aliquots before phosphate elution and in 0.4 ml aliquots during the elution. Radioactivity was counted by removing an aliquot (10 or 50 μl) of each fraction volume and mixing with scintillation fluid. Fractions containing the radioactive peak were pooled (M pool) and the volume was reduced to 0.2 ml by freeze-drying. After adjustment of the pH to 2 with 100% (v/v) trifluoroacetic acid (approx. 10 μl), the concentrated M pool was loaded on a C_{18} HPLC column (Vydac 208TP54, 4.6 mm $\times$ 250 mm), which was equilibrated with solvent H1 [5% (v/v) acetonitrile/95% (v/v) water/0.1% (v/v) trifluoroacetic acid] and running at a flow rate of 0.5 ml/min. After the injection, a gradient was applied that consisted of 5 min of solvent H1 and 65 min of a linear gradient to solvent H2 [70% (v/v) acetonitrile/30% (v/v) water/0.1% (v/v) trifluoroacetic acid]. Fractions were collected and counted for radioactivity. The radioactive fractions were pooled, concentrated to 25–50 μl in a Speed-Vac and stored at -80 °C.

Peptide sequencing was performed in the Protein Chemistry Laboratory of the University of Pennsylvania School of Medicine with a gas-phase microsequencer (Applied Biosystems 473A).

Statistics

All results presented in this report are means $\pm$ S.D. for six to ten samples from three to five separate experiments, unless indicated otherwise.

RESULTS

Purification of recombinant human 5LO

To perform the structural analysis of 5LO described below, a previously reported purification procedure [25,26] was modified to increase the total production and concentration of the purified 5LO. In this modified protocol, 5LO was expressed in *E. coli* cultured in a 20-litre fermenter containing super-rich medium. The enzyme was separated by batchwise Sepharose Q absorption, before final purification on an ATP-agarose column. Figure 1 shows one of the routine SDS/PAGE analyses after ATP-affinity chromatography. As demonstrated, the column separation was efficient, and fractions T3, T4, T5, T6 and T7 (eluted by ATP) contained significant amounts of 5LO with a purity of more than 95%. The fractions were stored separately and desalted immediately before labelling experiments (see the Experimental section for details).

Time course and dose-dependence of FSBA labelling

To examine the efficiency of FSBA labelling, 5LO was incubated with various amounts of FSBA at 30 °C for various periods. An aliquot of the reaction solution was then diluted 1:50 into an activity assay mixture and analysed in the presence and absence of ATP. As shown in Figure 2, the ATP-stimulated activity of 5LO decreased with increasing FSBA incubation time and concentration. An inhibition plateau was reached after 90 min of incubation (Figure 2A) and at an FSBA concentration of 0.5 mM (Figure 2B). Part of the inhibition was due to spontaneous inactivation of the enzyme during the incubation at 30 °C, because the 5LO activity in the vehicle control (Figure 2A) also

Figure 1 SDS/PAGE analysis of fractions from ATP column chromatography

Recombinant 5LO from 4.5 litres of *E. coli* culture was processed as described in the Experimental section and purified on an ATP-agarose column. Fractions from the column were analysed by SDS/PAGE [10–15% (w/v) gradient gel, 4 μ l per lane, Coomassie Blue staining] with a PhastSystem (Pharmacia). L1 and L2 are the fractions collected during the first cycle of loading and washing, M1–M4 are AMP elution fractions and T1–T12 are ATP elution fractions. The protein concentrations in T3, T4, T5, T6 and T7 were 0.26, 0.33, 0.48, 1.45 and 0.47 mg/ml respectively.

Figure 2 Time-dependent and concentration-dependent inhibition of 5LO by FSBA labelling

5LO (0.3–0.5 mg/ml) was incubated at 30 °C with 1 mM FSBA in 2% (v/v) DMSO (●, ■) or 2% (v/v) DMSO (○, □) for 0–120 min (A) or with 0–1 mM FSBA in 2% (v/v) DMSO for 120 min (B). The reaction mixtures were diluted 1:50 into assay mixtures and analysed for 5LO activity in the presence (○, ●) or absence (□, ■) of 10 mM ATP.

decreased (30–35%) with time. The inhibition by FSBA was apparently irreversible because a 1:50 dilution of the reaction solution into the assay mixture did not reverse the inhibition.

Figure 3 ATP protects 5LO from FSBA labelling inhibition

5LO (0.3–0.5 mg/ml) was incubated with 1 mM FSBA for 2 h in the presence of 0–100 mM ATP at 30 °C. The reaction solutions were diluted 1:50 and analysed for 5LO activity in the presence of 10 mM ATP. 5LO activity is plotted as a percentage of the control, which consisted of 5LO incubated with vehicle [2% (v/v) DMSO] only and analysed under the same conditions.

Specificity and stoichiometry of FSBA labelling

To examine the specificity of the FSBA labelling, increasing amounts of ATP were added to the FSBA labelling solution. As shown in Figure 3, the inhibition of 5LO caused by FSBA labelling was attenuated by the presence of ATP. At an ATP concentration of 50 mM (FSBA concentration 1 mM), approx. 80% of the FSBA inhibition was prevented. The requirement of a 50-fold excess of ATP to compete with the FSBA labelling is probably a consequence of the irreversibility of FSBA labelling.

Stoichiometry of the FSBA incorporation was analysed by incubation of 5'-*p*-[adenine-8-¹⁴C]FSBA with 5LO. To ensure that the non-covalently bound FSBA was completely removed after labelling, FSBA was quenched by 2-mercaptoethanol and 5LO was denatured with guanidinium chloride before separation on a gel-filtration column. With this procedure, the stoichiometry of incorporation was 1.4 mol of FSBA per mol of 5LO in the absence of ATP, and 0.4 mol per mol in the presence of 50 mM ATP (Table 1). Thus ATP competed with 1 mol of the incorporated FSBA.

When FSBA was not quenched with 2-mercaptoethanol before 5LO denaturation, 2.7 mol of FSBA was incorporated per mol of

Table 1 Stoichiometry of covalently bound FSBA

5LO (10–20 μ M) was incubated with 1 mM [14 C]FSBA at 30 °C for 120 min in the absence or the presence of 50 mM ATP. The reaction was terminated with or without the addition of 50 mM 2-mercaptoethanol. Non-covalently bound FSBA was removed by gel filtration in the presence of 5 M guanidinium chloride. The protein concentration and the radioactivity in the protein fractions were determined, and the molar ratio of FSBA to 5LO was calculated. The amount of FSBA that could be competed by ATP (last column) was calculated as the difference between the previous two columns. Incubations performed in the absence or the presence of Ca^{2+} were also compared. Results are means $\pm$ S.D. for two independent experiments, in each of which three gel-filtration fractions were analysed in duplicate.

Present during incubation			FSBA/5LO (mol/mol)		
EDTA (mM)	Ca^{2+} (mM)	Termination 2-Mercaptoethanol (mM)	No ATP	50 mM ATP	ATP competed
1	—	50	1.42 ± 0.19	0.38 ± 0.03	1.04
1	1.5	50	1.49 ± 0.07	0.39 ± 0.02	1.10
1	—	—	2.71 ± 0.31	0.72 ± 0.10	1.99
1	1.5	—	2.72 ± 0.21	0.69 ± 0.08	2.03

Figure 4 Photolabelling of 5LO with 2-azido-[32 P]ATP

5LO (0.06–0.1 mg/ml) was mixed with 50 μ M 2-azido-[γ - 32 P]ATP and irradiated at 254 nm for 0–120 s (A), or with 0–100 μ M 2-azido-[γ - 32 P]ATP and irradiated at 254 nm for 10 s (B). Incorporation of 2-azido-[γ - 32 P]ATP was estimated by separating 5LO from free ligand on SDS/PAGE and counting the radioactivity of the 5LO gel band (A) or both the 5LO and ligand gel bands (B).

5LO; ATP effectively competed with 2 mol of the incorporated FSBA (Table 1). This additional 1 mol of incorporated FSBA was probably associated with the exposure of reactive residues (lysine or tyrosine) during the denaturation.

Figure 5 Photolabelling of 5LO with 8-azido-[32 P]ATP

5LO (0.06–0.1 mg/ml) was mixed with 100 μ M 8-azido-[γ - 32 P]ATP and irradiated at 254 nm for 0–120 s (A), or with 0–100 μ M 8-azido-[γ - 32 P]ATP and irradiated at 254 nm for 90 s (B). Incorporation of 8-azido-[γ - 32 P]ATP was estimated by separating 5LO from free ligand on SDS/PAGE and counting the radioactivity of the 5LO gel band (A) or both the 5LO and ligand gel bands (B).

Previous studies have shown that Ca^{2+} is required for ATP stimulation of 5LO [13,14,20,21]. Thus Ca^{2+} was added to the labelling mixture and its effect on the stoichiometry of FSBA labelling was examined. As shown in Table 1, the presence of

Figure 6 Competition of 2-azido-ATP labelling with nucleotides

5LO (0.06–0.1 mg/ml) was mixed with 50 μ M 2-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and irradiated at 254 nm for 10 s in the presence of 0–5 mM ATP (**A**) or various nucleotides at 1 mM (**B**). Incorporation of 2-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was estimated by separating 5LO from free ligand on SDS/PAGE and counting the radioactivity of the 5LO gel band. The control sample (ctl) was labelled with 2-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the absence of other nucleotides.

0.5 mM free Ca^{2+} in the labelling mixture did not affect the FSBA incorporation or the ATP competition, suggesting that Ca^{2+} was irrelevant for FSBA binding.

Further sequencing of the FSBA-labelled peptide was not pursued because the commercially available $[\text{C}^{14}]\text{FSBA}$ had the radioisotope on the adenine ring. The ester bond between the benzoic acid and sugar moiety is unstable at $\text{pH} < 6$ and would have been cleaved during the peptide separation on HPLC, which uses a pH 2 solvent.

Labelling with 2-azido-ATP and 8-azido-ATP

Under UV irradiation, the azido group is converted into the highly reactive nitrene and attacks adjacent residues, forming a covalent bond between the nucleotide and the protein. We initially compared the labelling efficiency of 2-azido-ATP and 8-azido-ATP, because the two molecules have been shown to maintain different conformations (see the Discussion section). As shown in Figures 4 and 5, the labelling efficiency of 2-azido-ATP is significantly higher than that of 8-azido-ATP. With 2-azido-ATP labelling, the incorporation plateau was achieved after 10 s of UV irradiation (Figure 4A), whereas it was achieved after 90 s with 8-azido-ATP labelling (Figure 5A). At a probe concentration of 100 μ M, approx. 0.92 mol of 2-azido- $[\text{P}^{32}]\text{ATP}$ was incorporated per mol of 5LO (Figure 4B), but only 0.41 mol of 8-azido- $[\text{P}^{32}]\text{ATP}$ was incorporated per mol of 5LO (Figure 5B).

Table 2 Effect of labelling on 5LO activity

5LO (0.3–0.5 mg/ml) was labelled with FSBA (1 mM, 2 h), 2-azido-ATP (50 μ M, UV-irradiated for 90 s) or 8-azido-ATP (100 μ M, UV-irradiated for 90 s). UV-irradiated control was prepared by irradiating 5LO in buffer for 90 s. The labelling solutions were then diluted 1:100 in assay mixtures (containing 100 μ M arachidonic acid and 20 μ M phosphatidylcholine, but no Ca^{2+}) and analysed for 5LO activity in the absence and the presence of 10 mM ATP. Results are means $\pm$ S.D. for four separate experiments, each in duplicate, and are presented as percentages of the control, which had a specific activity of 4–6 nmol/ μ g.

5LO	ATP (mM)	Activity (% of control)
Untreated (control)	0	100
	10	319 $\pm$ 53
FSBA-labelled	0	37 $\pm$ 19
	10	54 $\pm$ 32
2-Azido-ATP-labelled	0	29 $\pm$ 7
	10	28 $\pm$ 8
8-Azido-ATP-labelled	0	25 $\pm$ 9
	10	36 $\pm$ 3
UV-irradiated	0	64 $\pm$ 14
	10	180 $\pm$ 29

Figure 7 Isolation of labelled peptides on a metal-chelating column

5LO (1.3 mg) was photoaffinity-labelled with 2-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and digested with Tos-Phe- CH_2Cl -trypsin. The digest (3 ml) was diluted 1:15 in buffer M1 (100 mM ammonium acetate, pH 5.9), and loaded on a 1.4 ml Al^{3+} -charged metal chelating column (iminodiacetic-acid-immobilized Sepharose 6B fast flow beads). After being washed with 15 ml of buffer M1, 5 ml of buffer M2 (0.5 M NaCl in buffer M1), and 5 ml of buffer M1, the column was eluted with 20 mM potassium phosphate in 100 mM ammonium acetate, pH 8.0. Radioactivity was counted in an aliquot of each fraction and is expressed as c.p.m./50 μ l of eluate.

All subsequent studies were therefore performed with 2-azido-ATP labelling only.

Specificity of 2-azido-ATP labelling

Addition of ATP to the 2-azido-ATP labelling solution dose-dependently decreased the incorporation of the label. As shown in Figure 6(A), ATP at concentrations of 1 and 5 mM decreased the 2-azido-ATP incorporation by 68% and 82% respectively. The addition of 1 mM ADP, AMP and GTP showed approximately the same competitive efficiency as ATP, although CTP and UTP were 30% and 10% respectively less competitive than ATP (Figure 6B). At 10 mM, all the nucleotides (ATP, ADP, AMP, GTP, CTP and UTP) competed similarly and decreased

Figure 8 Separation of peptides by HPLC

5LO (1.3 mg) was labelled with 2-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the absence (**A, B**) and in the presence (**C, D**) of 10 mM ATP. The labelled 5LO was digested with Tos-Phe- CH_2Cl -trypsin and separated on a metal-chelating column as described in the legend to Figure 7. The radioactive pool was concentrated and injected into a C_{18} column (Vydac 208TP54, 4.6 mm $\times$ 250 mm) equilibrated with 5% (v/v) acetonitrile/95% (v/v) water/0.5% (v/v) trifluoroacetic acid at a flow rate of 0.5 ml/min. At 5 min after the injection, a 65 min linear gradient was applied to reach 70% (v/v) acetonitrile/30% (v/v) water/0.5% (v/v) trifluoroacetic acid. (**A, C**) On-line UV absorption of eluate at 214 nm (solid line) and 280 nm (dotted line). (**B, D**) Radioactivity of the eluate as measured from collected fractions. See text for details of sample pools I(N), II(N), I(T) and II(T).

the incorporation by 87% (results not shown). The presence of 0.5 mM Ca^{2+} in the labelling mixture did not affect 2-azido-ATP incorporation or ATP competition, in agreement with the results from labelling with FSBA (Table 1).

Labelling of 5LO with FSBA or with 2-azido-ATP or 8-azido-ATP prevented the enzyme from being further labelled by 2-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, indicating that the 2-azido-ATP labelling site was similarly occupied by FSBA and 8-azido-ATP. Incorporation of 2-azido-ATP or 8-azido-ATP also inhibited the ATP-stimulated activity of 5LO irreversibly, as was observed with FSBA labelling (Table 2). Part of the inhibition was caused by inactivation of the enzyme during the 90 s of UV irradiation because the irradiated control lost 40% of both basal and ATP-stimulated activity (Table 2).

Isolation of labelled peptide

2-Azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ -labelled 5LO was completely digested by Tos-Phe- CH_2Cl -trypsin; the peptides were separated on two columns. The first column was an Al^{3+} -charged metal chelating column, which binds to phosphate-containing peptides as well as

to nucleotides at low pH (5.9) [41]. Figure 7 shows the separation of the labelled peptides, where the radioactive label was bound to the column and retained during extensive washing; it was eluted by 20 mM phosphate buffer at pH 8.

The second column was a C_{18} HPLC column loaded with the concentrated eluate from the metal-chelating column. With an acetonitrile gradient of 5–70% (v/v) and with a gradient increasing at 1%/min, two major peaks were found in the chromatogram and both contained radioactivity (for example, see Figures 8A and 8B). The early-eluting peak (retention time 8–12 min) was identified as the free-nucleotide peak, as demonstrated in a separate control experiment. The second major peak (retention time 43.0–44.8 min) contained two adjacent peaks that were not well separated. The retention times of the two peaks were 43.0–44.0 and 44.0–44.8 min respectively.

Peptide sequencing

Three batches of labelling and sequencing experiments were performed. In the first two rounds, the peptide fractions that were eluted from the C_{18} column at a retention time of 43.0–

Table 3 Sequences of 2-azido-ATP-labelled 5LO peptides

5LO was labelled with 2-azido-[γ - 32 P]ATP and digested by Tos-Phe-CH₂Cl-trypsin. Labelled peptides were isolated by a metal-chelating column and a C₁₈ HPLC column. Radioactive peptide fractions were pooled and analysed on a gas-phase microsequencer. Results are from three separate batches of sequencing analysis: (A) and (B) were from the first and second round of analysis; (C), (D) and (E) were obtained from the third round of analysis on sample pools I(N), II(N) and II(T) respectively. See the text for details.

(A)

Cycle	Residue	Quantity (pmol)	Residue	Quantity (pmol)
1	K	93.1	F	30.1
2	Y	90.1	M	24.8
3	X	—	H	10.0
4	L	89.2	M	15.3
5	N	58.7	F	16.8
6	D	39.1	Q	11.4
7	D	55.5	S	3.7
8	W	16.9	S	3.2
9	Y	41.8	X	—
10	L	42.1	N	7.7
11	K	15.1	D	8.4
12			F	7.9
13			A	6.2
14			D	5.9
15			F	5.7
16			E	3.4
17			K	3.5

(B)

Cycle	Residue	Quantity (pmol)	Residue	Quantity (pmol)	Residue	Quantity (pmol)
1	K	113.4	F	10.8	N	3.3
2	Y	116.7	M	10.3	L	12.2
3	X	—	H	2.6	A	12.4
4	L	123.1	M	7.7	N	10.7
5	N	88.6	F	6.7	K	7
6	D	87.5	Q	6.4	X	—
7	D	78.6	S	1.0	V	7.9
8	W	34.9	S	1.0	P	4.2
9	Y	76.0	X	—	I	4.9
10	L	56.7	N	2.5	A	6
11	K	49.2	X	—	I	4.8
12			F	2.7	Q	4.3
13			A	3.9		
14			D	3.1		
15			F	3.1		
16			E	1.6		
17			K	1.5		

(C)

Cycle	Residue	Quantity (pmol)	Residue	Quantity (pmol)
1	K	75.4	F	17.0
2	Y	67.2	M	14.7
3	X	—	H	4.9
4	L	81.2	M	11.1
5	N	43.2	F	10.8
6	D	37.4	Q	8.7
7	D	49.5	S	2.5
8	W	12.0	S	2.3
9	Y	34.4	X	—
10	L	51.9	N	5.1
11	K	25.5	X	—
12			F	5.3
13			A	4.2
14			D	4.2
15			F	4.5
16			E	2.4
17			K	3.2

Table 3 (cont.)

(D)

Cycle	Residue	Quantity (pmol)	Residue	Quantity (pmol)
1	K	115.9	F	13.4
2	Y	102.6	M	10.1
3	X	—	H	3.0
4	L	144.8	M	9.2
5	N	67.7	F	7.4
6	D	62.6	Q	7.0
7	D	75.2	S	1.6
8	W	30.5	S	1.7
9	Y	65.2	X	—
10	L	71.1	N	3.7
11	K	38.1	X	—
12			F	4.0
13			A	3.2
14			D	3.1
15			F	3.3
16			E	2.0
17			K	2.6

(E)

Cycle	Residue	Quantity (pmol)	Residue	Quantity (pmol)
1	K	48.1	F	4.3
2	Y	43.0	M	2.5
3	X	—	H	1.0
4	L	42.8	M	1.9
5	N	21.5	F	1.7
6	D	17.7	Q	1.5
7	D	23.0	X	—
8	W	5.8	X	—
9	Y	18.8	X	—
10	L	21.0	X	—
11	K	10.3	X	—
12			F	0.8
13			A	0.9
14			X	—
15			F	0.7
16			E	0.7
17			X	—

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Rat 5LO ELGEIYLVKIEKRRYRLHDDWYLYITLKTTPH---DYIEFPCYRWITGEG
Mouse 5LO ELGEIYLVKIEKRRYRLHDDWYLYITLKTTPHG--DYIEFPCYRWITGEG
Hamster5LO ELGEIQLVRIEKRKYRLHDDWYLYITLKTTPH---DYIEFPCYRWITGEG
Human 5LO ELGEIQLVRIEKRKYRLHDDWYLYITLKTTPHG--DYIEFPCYRWITGEG
Human 15LO YLGPIILFVRLRKRHLKDDAWFCNWSVQGPAG-DEVRFPCYRWVGGNG
Rabbit15LO YLGSLLFVRLRKRHLKDDAWFCNWSVQALGAEDKYWFPCYRWVGGNG
Rat 12LO YLGPIILFVRLRKRHLKDDAWFCNWSVQGPAG-DEVRFPCYRWVGGNG
Human 12LO DLGLLQFVRLRKRHLKDDAWFCNWSVQGPAG-DEVRFPCYRWVGGNG
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Rat 5LO VLNYSKAMENLFINRFMHMFQSSWHDPADEFKIFVKISNTISERVKNHWQ
Mouse 5LO VLNYSKAMENLFINRFMHMFQSSWHDPADEFKIFVKISNTISERVKNHWQ
Hamster5LO VLNYSKAMENLFINRFMHMFQSSWHDPADEFKIFVKISNTISERVKNHWQ
Human 5LO VLNYSKAMENLFINRFMHMFQSSWHDPADEFKIFVKISNTISERVKNHWQ
Human 15LO EVSLAKGLADLAIKDSLNLVT-CWKDLDDFNRIFWCGQSKLAERVDSWK
Rabbit15LO EASLAWGLADLAIKDSLNLVT-PWKTLDFFNRIFWCGQSKLAERVDSWK
Rat 12LO EASQVIGVMDTVVNFPIINTVT-CWKSLLDFNCFVPSGHTKMAERVNSWK
Human 12LO EWTLKAGALEMALKRVYTLIS-SWNCLEDFDQIFWQGSALAEKVRQCWQ
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Figure 9 Comparison of sequences of eight lipoxigenases in the regions of the labelled peptides

Lipoxigenase sequences were aligned by a Protein Information Resource (PIR) multiple alignment program with CLUSTAL W, version 1.8. Only the regions adjacent to the labelled peptides (in bold and underlined) are shown. The PIR numbers for the first and the fourth to eighth lipoxigenases are A30882, DAHUAL, A31349, JQ0018, S30051 and A38283 respectively. The GenBank® protein accession numbers for mouse and hamster 5LO are AAC37673.1 and AAA85257.1 respectively. Completely conserved residues are marked by asterisks, partially conserved residues up to 51% are marked by full stops and partially conserved residues up to 82% by colons under the alignment.

44.8 min were pooled and sequenced together. As shown in Tables 3(A) and 3(B), these two sequencing analyses revealed two peptides in common: one had the sequence KYXLNDDWYLK (in single-letter amino acid code), and the other FMHMFQSSXNDFADFEK. These sequences are equivalent to the cDNA-derived 5LO peptide sequences [29,31] 73–83 and 193–209. The missing (or modified) residue (X) in the first peptide is Trp-75 and in the second is Trp-201. The relative abundances of the two peptides were 3:1 (in the first round of sequencing analysis) and 10:1 (in the second round) respectively, indicating that the first peptide was predominantly labelled. In the second round of sequencing, a third and least abundant peptide also appeared, NLANKXVPVIAIQ, which corresponded to 5LO peptide sequence 315–326, with the modified residue being Ile-320.

To ensure that the peptides were specifically labelled at the ATP-binding site, a third batch of sequencing was performed with labelling of 5LO in two separate tubes. Each tube contained the same amounts of 5LO and 2-azido-[γ - 32 P]ATP but one had an additional 10 mM ATP. The same UV irradiation and column separations were performed for both samples. In this round of sequencing, the fractions from the C_{18} column separation were pooled according to the positions of the two peaks (Figure 8). Pool I contained eluates at the retention time of 43.0–43.8 min, and pool II those at 44.2–44.8 min. Four pools were obtained from the last column separations: Pool I(N) and Pool II(N) from the separation of the normal labelling sample, and Pool I(T) and Pool II(T) from the separation of ATP-competed labelling sample.

As shown in Figure 8, the intensity of the peptide peaks at the retention time of 43–44.8 min was significantly attenuated in the ATP-competed-labelling sample [compare Figures 8(C) and 8(D) with Figure 8(A) or Figure 8(B)]. Consequently, the amount of peptides in Pool I(T) was too small to be sequenced, as determined by the MS estimation performed before each sequencing analysis. Pools I(N), II(N) and II(T) were sequenced; the results are shown in Tables 3(C), 3(D) and 3(E) respectively. The ratio between peptides KYXLNDDWYLK and FMHMFQSSXNDFADFEK was approx. 4 in pool I(N), approx. 9 in pool II(N) and approx. 11 in pool II(T). Thus ATP attenuated the labelling of the two peptides relatively proportionately.

DISCUSSION

The fact that 5LO does not contain a consensus ATP-binding or nucleotide-binding sequence has raised a question about whether or not the enzyme does indeed bind ATP. This study shows that the binding does exist and occurs at a specific site. The evidence for this conclusion is as follows: (1) two different types of ATP analogue, FSBA and azido-ATP, bound to and labelled 5LO; (2) the labelling in both cases was quantitative (stoichiometric) and specific (competed by ATP); and (3) the covalent nucleotide labelling was found reproducibly on specific peptide sequences of 5LO, Lys-73–Lys-83 and Phe-193–Lys-209. Because other nucleotides, ADP, AMP, GTP, CTP and UTP, also competed with the labelling, we consider the site to be a general nucleotide-binding site rather than a strict ATP-binding site. Ca^{2+} was not required for, and could not compete with, the labelling. Thus the nucleotide-binding and Ca^{2+} -binding sites apparently do not overlap, and a Ca^{2+} –ATP complex is not required for preferential binding.

The labelled peptides, Lys-73–Lys-83 and Phe-193–Lys-209, are distant from each other in the primary sequence of 5LO. They could either represent two distinct nucleotide-binding sites

of 5LO or be adjacent in the three-dimensional structure of the enzyme to comprise a single binding site. We felt that the latter is more likely, because (1) stoichiometric analyses of FSBA and 2-azido-ATP labelling both showed that only 1 mol of the ATP analogues was bound to one mol of 5LO, and (2) the labelling occurred predominantly on Lys-73–Lys-83 and ATP competed with the labelling of both peptides proportionately.

A third fragment of 5LO (N^{315} LANKIVPIAIQ 326) was found labelled in one of the sequence analyses. However, the abundance of the labelled peptide was low and the labelling was not found reproducibly in other rounds of sequencing. Therefore this segment might be located near, but not in, the binding site; its chance of reacting with nitrene was low during photolysis.

A considerable difference was found in the efficiency of labelling by 2-azido-ATP and 8-azido-ATP. This difference is probably due to the shape (or contour) of the binding site of 5LO. 8-Azido-ATP has a C-8 substitution on the adenine ring, which has been shown to restrict the molecule to a *syn* conformation, i.e. the purine and sugar rings are on the same side of the N-glycosidic bond [42]. In contrast, C-2 substitution does not seem to affect the rotation of the bond. Hence 2-azido-ATP has both *anti* and *syn* conformations (the ratio of *anti* to *syn* is 60/40) [43], as found in natural nucleotides [44]. The selectivity of 5LO towards 2-azido-ATP would suggest that the binding site in 5LO is better suited for nucleotides in the *anti*, rather than in the *syn*, conformation. In previous experiments we have found that polycationic molecules, such as histone and polylysine, could increase the photoincorporation of 8-azido-ATP into 5LO (results not shown). Therefore a conformational change in the nucleotide-binding site of 5LO could possibly be induced under certain conditions.

Other lipoxygenases, such as 12LO and 15LO, cannot be purified by ATP column chromatography, although they have substantial sequence similarity to 5LO. It was therefore interesting to examine whether or not these lipoxygenases contain sequence fragments similar to that of the labelled peptides found in 5LO. A multiple alignment of sequences of eight lipoxygenases (Figure 9) shows that 12LOs and 15LOs have rather different sequences in the regions where the labelled peptides are located, but all 5LOs (from rat, mouse, hamster and human) have similar sequences. The alignment also showed that Trp-75, Asn-77 and Asn-202 in the labelled peptides of human 5LO are replaced by different residues in other 5LOs, indicating that these residues might not be critical for ATP binding, even though Trp-75 was labelled by 2-azido-ATP. In addition, Asp-78, Trp-80, Trp-201, Asp-206 and Phe-207 are conserved in all three types of lipoxygenase, suggesting that they might have a common structural significance in these proteins. The crystal structure has been solved for rabbit 15LO [45] but not for any of the 5LOs. The corresponding fragments in 15LO are found on the surface of the protein.

The sequences of the labelled peptides have been compared with proteins registered in two protein databanks, Swiss-Prot and Protein Information Resource. The analysis showed that no other protein contains segments identical with the peptides. However, marginal similarity (for definition see [46]) was found between the peptides and a number of proteins. More than one-third of them are nucleotide-binding or nucleic-acid-binding proteins. Lining up the matched segments in the nucleotide-binding or nucleic-acid-binding proteins with the labelled peptides showed that some residues (Lys-73, Leu-76, Leu-82, Lys-83, Ser-199, Asp-203, Asp-206 and Glu-208) in the labelled peptides were more conserved than others. Further mutagenesis and binding studies are required to establish the binding site pattern residing in these peptides and the surrounding region.

Regulation of 5LO by ATP and nucleotides is likely to occur in the cell nucleus, because the enzyme is located in the nuclei of various cell types in the quiescent state [28,47,48]. Such regulation might reflect the function of the enzyme in the nucleus, which is so far still unclear. Various studies have shown that 5LO is involved in cell proliferation [2–7], but the association of this function and the nuclear location of the enzyme has not been demonstrated. Further intracellular study of 5LO deficient in nucleotide binding might shed some light in this regard.

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