Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 8383–8387, August 1995
Biochemistry

Leukotriene A_4 hydrolase: Mapping of a henicosapeptide involved in mechanism-based inactivation

(suicide inactivation/inflammation)

MARTIN J. MUELLER, ANDERS WETTERHOLM, MARTINA BLOMSTER, HANS JÖRNVALL, BENGT SAMUELSSON, AND JESPER Z. HAEGGSTRÖM*

Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden

Contributed by Bengt Samuelsson, May 1, 1995

ABSTRACT **Leukotriene A_4 (LTA_4) hydrolase [(7*E*,9*E*,11*Z*,14*Z*)-(5*S*,6*S*)-5,6-epoxyicosa-7,9,11,14-tetraenoate hydrolase; EC 3.3.2.6] is a bifunctional zinc metalloenzyme which converts LTA_4 into the chemotactic agent leukotriene B_4 (LTB_4). Suicide inactivation, a typical feature of LTA_4 hydrolase/aminopeptidase, occurs via an irreversible, apparently mechanism-based, covalent binding of LTA_4 to the protein in a 1:1 stoichiometry. Differential lysine-specific peptide mapping of unmodified and suicide-inactivated LTA_4 hydrolase has been used to identify a henicosapeptide, encompassing the amino acid residues 365-385 of human LTA_4 hydrolase, which is involved in the binding of LTA_4, LTA_4 methyl ester, and LTA_4 ethyl ester to the native enzyme. A modified form of this peptide, generated by lysine-specific digestion of LTA_4 hydrolase inactivated by LTA_4 ethyl ester, could be isolated for complete Edman degradation. The sequence analysis revealed a gap at position 14, which shows that binding of the leukotriene epoxide had occurred via Tyr-378 in LTA_4 hydrolase. Inactivation of the epoxide hydrolase and the aminopeptidase activity was accompanied by a proportionate modification of the peptide. Furthermore, both enzyme inactivation and peptide modification could be prevented by preincubation of LTA_4 hydrolase with the competitive inhibitor bestatin, which demonstrates that the henicosapeptide contains functional elements of the active site(s). It may now be possible to clarify the molecular mechanisms underlying suicide inactivation and epoxide hydrolysis by site-directed mutagenesis combined with structural analysis of the lipid molecule, covalently bound to the peptide.**

Leukotriene (LT) B_4 is a product of activated leukocytes and has potent autocrine, chemotactic, and chemokinetic properties (1). The final step in the biosynthesis of LTB_4 is catalyzed by the enzyme LTA_4 hydrolase [(7*E*,9*E*,11*Z*,14*Z*)-(5*S*,6*S*)-5,6-epoxyicosa-7,9,11,14-tetraenoate hydrolase; EC 3.3.2.6), which is a 69-kDa soluble zinc metalloprotein with no apparent structural similarity to other epoxide hydrolases (2). In addition to its well-known epoxide hydrolase activity—i.e., the conversion of LTA_4 into LTB_4, LTA_4 hydrolase possesses an aminopeptidase activity toward synthetic chromogenic amides, opioid peptides, and arginyl tripeptides (3–6), but the endogenous peptidase substrate is still not known. Both activities are dependent on the intrinsic zinc atom and are competitively inhibited by bestatin (7) and captopril (8), classic inhibitors of aminopeptidases and angiotensin converting enzyme, respectively.

Previous work has identified several important elements of the active site(s) corresponding to the two enzyme activities. For instance, the intrinsic zinc atom has been shown to be catalytic and complexed to His-295, His-299, and Glu-318. Anions selectively stimulate the peptidase activity, apparently via an anion binding site, and site-directed mutagenesis studies have identified Glu-296 as a catalytic residue which presumably acts as a general base in the peptidase reaction (9). Much less is known about the mechanism for the epoxide hydrolase activity.

Typically, LTA_4 hydrolase is covalently modified and inactivated during catalysis by its endogenous lipid substrate LTA_4, a process commonly referred to as suicide inactivation (10, 11). Since LTA_4 hydrolase has been proposed to be the rate-limiting enzyme in cellular LTB_4 biosynthesis (12), suicide inactivation may be an important mechanism for the overall regulation of this biosynthetic pathway *in vivo*.

A number of studies have dealt with suicide inactivation of LTA_4 hydrolase (10, 11, 13). The data collected satisfy several criteria which define a mechanism-based process (14). Thus, after treatment of LTA_4 hydrolase with LTA_4 or with LTA_4 methyl ester, both catalytic activities are lost simultaneously in a time-dependent process obeying pseudo-first-order kinetics (15). Furthermore, the partition ratio between turnover and inactivation of an LTA_4–enzyme complex has been calculated to 129 ± 16 (15). Active-site specificity of the process has been demonstrated by protection of the enzyme with the competitive inhibitors captopril and bestatin, and analysis with electrospray mass spectrometry has revealed that suicide inactivation with LTA_4 and LTA_4 methyl ester occurs predominantly in a 1:1 stoichiometry between lipid and protein, with little modification of secondary sites (15, 16).

Although suicide inactivation of LTA_4 hydrolase has been well characterized by kinetic analysis and mass spectrometry, the molecular mechanisms and structural determinants of the process are still unknown. We report here the identification of a henicosapeptide which is critically involved in the covalent binding of LTA_4, LTA_4 methyl ester, and LTA_4 ethyl ester during suicide inactivation of LTA_4 hydrolase.

EXPERIMENTAL PROCEDURES

Materials. [14,15-^{3}H]LTA_4 methyl ester (specific activity 42 Ci/mmol; New England Nuclear; 1 Ci = 37 GBq), LTA_4 ethyl ester (Merck Frosst Labs, Pointe Claire, QC, Canada), and LTA_4 methyl ester (Biomol, Plymouth Meeting, PA) were used. Alkaline hydrolysis of LTA_4 ethyl ester was carried out as described (17). L-Alanine *p*-nitroanilide, dithiothreitol, iodoacetic acid sodium salt, and bestatin were purchased from Sigma, and CNBr was from Aldrich. PD-10 and Nick gel filtration columns were from Pharmacia. Glu-specific protease (500–1000 units/mg; Sigma) was dissolved at 2.5 μg/ml in 10 mM Tris·HCl (pH 8). Lys-C protease (150 units/mg, Boehringer) was dissolved in 1 mM HCl and stored up to 4 weeks at −20°C. Human leukocyte LTA_4 hydrolase and recombinant human LTA_4 hydrolase were purified as described (18).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: LTA_4 and LTB_4, leukotrienes A_4 and B_4.
*To whom reprint requests should be addressed.

Enzyme Assays. Epoxide hydrolase activity was determined at room temperature from incubations of LTA_4 hydrolase in 50 mM Tris·HCl at pH 8.0 (0.1 ml, enzyme at 5–10 μg/ml) with 1.5 μl of LTA_4 lithium salt in tetrahydrofuran (2.4–2.8 nmol/μl). Reactions were quenched after 15 s with 2 volumes of methanol containing prostaglandin B_1 (500 ng) as internal standard and LTB_4 was quantified by HPLC as described (19). The peptidase activity was determined spectrophotometrically in 50 mM Tris·HCl, pH 7.5/100 mM NaCl (20).

Inactivation Experiments. LTA_4 hydrolase (200 μg; 2.9 nmol) in 2 ml of 10 mM Tris·HCl (pH 8) was incubated with LTA_4, LTA_4 methyl ester, or LTA_4 ethyl ester (10 μl; 2.4–2.6 nmol/μl in tetrahydrofuran) for 20–30 min at room temperature. The addition of the leukotriene epoxides was repeated four times (final concentration, 60–65 μM). The concentration of tetrahydrofuran never exceeded 2.5% of the final volume and did not influence the enzymatic reactions. A portion of the reaction mixture (100 μl) was gel-filtered on a PD-10 column to remove reversibly bound lipids, and the protein fraction was used for quantitation of the residual epoxide hydrolase and peptidase activities. The remaining enzyme was used for proteolytic digestion and peptide mapping. In experiments with bestatin, LTA_4 hydrolase was preincubated with 2 mM inhibitor for 60 min prior to addition of the leukotriene epoxides.

Cleavage of Labeled LTA_4 Hydrolase with Glu-Specific Protease and CNBr. Human leukocyte LTA_4 hydrolase (600 μg in 2.5 ml of 10 mM Tris·HCl at pH 8) was incubated with unlabeled LTA_4 methyl ester (20 nmol) plus [^{3}H]LTA_4 methyl ester (1.8×10^6 dpm) for 10 min at room temperature. The mixture was gel-filtered on a PD-10 column, applied to a Pharmacia Mono Q (HR 5/5) anion-exchange column, and eluted with a linear gradient of 0–0.5 M KCl. Limited proteolysis of labeled LTA_4 hydrolase (75 μg of enzyme, 133 dpm/μg) with Glu-specific endoprotease (0.5 μl, 2.5 μg/μl) was performed at room temperature for 5 min in 10 μl 10 mM Tris·HCl (pH 8). The reaction was stopped by boiling (5 min) in loading buffer and the resulting peptides were separated by SDS/PAGE (resolving gel, 15% acrylamide; stacking gel, 5% acrylamide) and stained with Coomassie brilliant blue. Radiolabeled fragments were made visible by autoradiography for 9 days with Hyperfilm-MP (Amersham). CNBr cleavage was performed in 70% formic acid at room temperature for 24 hr. Only a limited fragmentation of LTA_4 hydrolase was observed after incubation in 70% formic acid at room temperature overnight.

Cleavage of Untreated and of Suicide-Inactivated LTA_4 Hydrolase with Lys-C Protease. Inactivated/untreated enzyme was gel-filtered on a PD-10 column, concentrated in a Centricon 10 unit (Amicon), dried under N_2, and dissolved in 75 μl of 6 M guanidinium chloride/0.4 M Tris·HCl/2 mM EDTA, pH 8.15. The denatured enzyme was reduced by incubation with 30 mM dithiothreitol for 30 min at 38°C under argon and carboxymethylated with 100 mM sodium iodoacetate for 10 min in the dark at room temperature. Excess reagents were removed by gel filtration (Nick column) into Lys-C digestion buffer (1.5 M deionized urea/50 mM borate buffer, pH 8) and LTA_4 hydrolase was cleaved overnight with Lys-C (1.5 μg/500 μl) at room temperature. Peptides were separated by reversed-phase HPLC on a Vydac column (218TP54; C_{18} resin, 5-μm pore size; column dimensions, 4.6 mm × 250 mm) by use of a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Sequence degradations were carried out with Applied Biosystems 470A and 477A instruments or a MilliGen 6600 Prosequencer. Phenylthiohydantoin derivatives were analyzed separately (470A) or with on-line HPLC systems (477A and 6600).

RESULTS AND DISCUSSION

Specificity of Covalent Binding of [^{3}H]LTA_4 Methyl Ester to LTA_4 Hydrolase. Initial work was carried out to delineate a region of LTA_4 hydrolase possibly involved in covalent binding of LTA_4. Thus, enzyme was exposed to [^{3}H]LTA_4 methyl ester (3×10^6 dpm/mg) and subjected to proteolytic and chemical cleavage. Digestion of labeled enzyme under mild conditions with a Glu-specific protease, which leads to a limited fragmentation of the protein, resulted in one labeled and one unlabeled major peptide fragment, with $M_r \approx 40{,}000$ and 30,000, respectively (Fig. 1). From this result we concluded that covalent binding of LTA_4 methyl ester was not spread randomly along the polypeptide chain. Furthermore, cleavage of labeled enzyme with CNBr generated a major tritiated fragment with $M_r \approx 30{,}000$ (data not shown). Considering the distribution of Met in the protein, and the specificity of CNBr, we tentatively identified this fragment as a central peptide spanning residues Glu-271 to Met-520.

Differential Lysine-Specific Peptide Mapping of Unmodified and Suicide-Inactivated LTA_4 Hydrolase. LTA_4 hydrolase (100 μg/ml) was treated with five consecutive additions of LTA_4 (13 μM), at 20- to 30-min intervals, to obtain suicide-inactivated enzyme. The modified protein was separated from noncovalently bound hydrolysis products of LTA_4 by gel filtration. After digestion with Lys-C protease, the resulting peptide mixture was resolved by HPLC on a C_{18} column. The absorbance was monitored at 214 nm and 280 nm to allow detection of both peptide bonds and aromatic amino acids, respectively (Fig. 2). In parallel experiments, identical peptide digests were prepared from suicide-inactivated and from unmodified enzyme for differential analysis by HPLC. The chromatographic pattern and the relative intensities of individual peptide peaks were highly reproducible in separations from no less than 14 cleavages of untreated LTA_4 hydrolase. However, HPLC analysis of peptides generated from suicide-inactivated enzyme revealed that the intensity of one peak was always reduced in the peptide map (Fig. 2). This peptide peak, denoted K21 (from its number of amino acid residues; see below), was eluted at 33% acetonitrile from the HPLC column.

Structure of Peptide K21. Material that was eluted under the peak, which was reduced in digests of suicide-inactivated enzyme, was collected and subjected to Edman degradation. Sequence analysis revealed the presence of a peptide (K21) spanning 21 residues from Leu-365 to Lys-385 (Fig. 3). Notably, peptide K21 could be distinguished from a smaller, partially resolved peptide by its absorbance at 280 nm (see Fig. 2). The contaminating peptide, encompassing residues Gly-83 to Lys-96 as determined by amino acid sequence analysis,

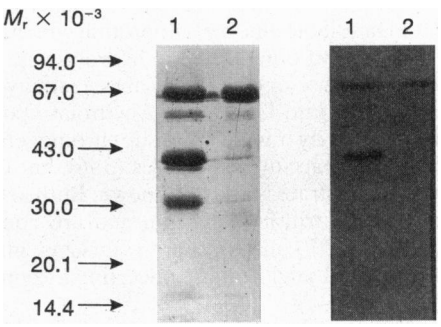

FIG. 1. Specific incorporation of [^{3}H]LTA_4 methyl ester into a proteolytic fragment of LTA_4 hydrolase. LTA_4 hydrolase was treated with [^{3}H]LTA_4 methyl ester for 10 min at room temperature and cleaved with Glu-specific protease at room temperature for 5 min. Peptides were separated by SDS/PAGE (*Left*) and labeled fragments were made visible by autoradiography (*Right*). Results obtained with cleaved and uncleaved labeled enzyme are shown in lanes 1 and 2, respectively.

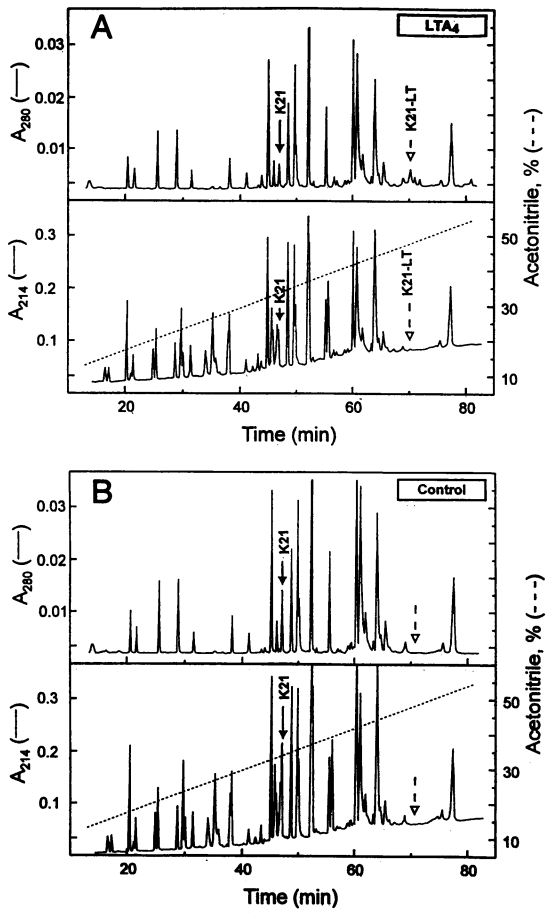

FIG. 2. Reversed-phase HPLC analysis of peptides generated by Lys-specific proteolysis of suicide-inactivated (*A*) and untreated (*B*) LTA_4 hydrolase. Enzyme (100 μg/ml) was incubated with LTA_4 (five additions of 13 μM) for 30 min at room temperature, carboxymethylated, and cleaved with Lys-C. Untreated LTA_4 hydrolase (control) was processed and cleaved under identical conditions. The resulting peptides were separated by reversed-phase HPLC and the absorbance was recorded at 214 nm and 280 nm. Solid arrows indicate the position of peptide K21 (see text), and open arrows the position of peptide K21-LT.

contains no tyrosine or tryptophan residues. Hence, this peptide, retained in peptide maps of suicide-inactivated enzyme, was characterized by a peak at 214 nm without a corresponding absorbance at 280 nm (see Fig. 2).

Sequence of peptide K21

```
Leu-Val-Val-Asp-Leu-Thr-Asp-Ile-Asp-Pro-Asp-Val-
365 366 367 368 369 370 371 372 373 374 375 376
Ala-Tyr-Ser-Ser-Val-Pro-Tyr-Glu-Lys
377 378 379 380 381 382 383 384 385
```

Sequence of the modified peptide K21-LTet

```
Leu-Val-Val-Asp-Leu-Thr-Asp-Ile-Asp-Pro-Asp-Val-
365 366 367 368 369 370 371 372 373 374 375 376
Ala-(*)-Ser-Ser-Val-Pro-Tyr-Glu-Lys
377 378 379 380 381 382 383 384 385
```

FIG. 3. Amino acid sequence analysis of peptides K21 and K21-LTet. A single peptide, denoted K21, was lost in peptide maps of suicide-inactivated LTA_4 hydrolase in proportion to enzyme inactivation. This peptide was collected and subjected to Edman degradation. Treatment of native enzyme with LTA_4 ethyl ester generated a modified form of peptide K21, referred to as K21-LTet, which was also recovered for amino acid sequence analysis. Numbering of amino acids refers to the corresponding position in the primary structure of LTA_4 hydrolase, excluding the first Met. The star represents a gap in the sequencer identifications.

Correlation Between Loss of Enzyme Activity and Loss of Peptide K21. To determine the degree of inactivation achieved after multiple additions of LTA_4, modified LTA_4 hydrolase was assayed for residual peptidase activity. This activity, expressed in percent of the activity measured with untreated control enzyme, was then compared with the presence of peptide K21 in the corresponding peptide map. The presence (or loss) of K21 in samples of suicide-inactivated LTA_4 hydrolase was calculated from the peak height at 280 nm in peptide maps. Values were normalized with respect to neighboring peaks to compensate for variations in recovery and were expressed in percent of the corresponding peak height obtained with untreated control enzyme. The loss of enzyme activity correlated well with the loss of peptide K21 in every experiment, within the limits of experimental error (Table 1), which indicates that the henicosapeptide is involved in covalent binding of the substrate LTA_4. A comparable change in relative intensity was not observed for any other fraction.

Generation and Identification of a Modified Peptide K21. The involvement of peptide K21 in covalent modification of LTA_4 hydrolase was further supported by the appearance of a novel peptide in digests of suicide-inactivated enzyme. Thus, material with strong absorption at 280 nm was eluted at 48% acetonitrile. In view of the high extinction coefficient of a conjugated triene, the exceptionally high A_{280}/A_{214} ratio of this peak strongly suggested the presence of a leukotriene moiety (Figs. 2 and 4). Consequently, the peptide corresponding to this peak was denoted K21-LT. Hydrolysis products of LTA_4 were eluted at a different percentage of acetonitrile and did not account for the high absorbance at 280 nm. Although the recovery of K21-LT was poor (compare peak size at 214 nm, Fig. 2) enough material could be collected to trace the N-terminal amino acid sequence, which was in agreement with the corresponding sequence of K21. Hence, suicide inactivation of LTA_4 hydrolase is accompanied by a shift of peptide K21 to the lipophilic region of the chromatogram, where it appears as peptide K21-LT (Fig. 2). In addition, several minor peaks were eluted after K21-LT. Although none of these peaks was positively identified, it seems likely that they represent

Table 1. Correlation between residual peptidase activity and presence of peptide K21 after inactivation with LTA_4 and LTA_4 methyl and ethyl esters

Inactivating compound	Residual peptidase activity, %	Amount of peptide K21, %
LTA_4	30	32
	21	33
	19	30
	40	40
	35	52
	42	46
	56	50
	(34.7 ± 12.9)	(40.4 ± 9.1)
LTA_4 methyl ester	14	15
	15	21
	23	23
	(17.3 ± 4.9)	(19.6 ± 4.2)
LTA_4 ethyl ester	22	22
	10	11
	10	9
	10	14
	(13.0 ± 6.0)	(14.0 ± 5.1)

The peptidase activity and amount of peptide K21 detected in samples of suicide-inactivated LTA_4 hydrolase are expressed in percent of values obtained with untreated control enzyme. Values in parentheses are mean ± SD for $n = 7$ (LTA_4), $n = 3$ (LTA_4 methyl ester), and $n = 4$ (LTA_4 ethyl ester) samples.

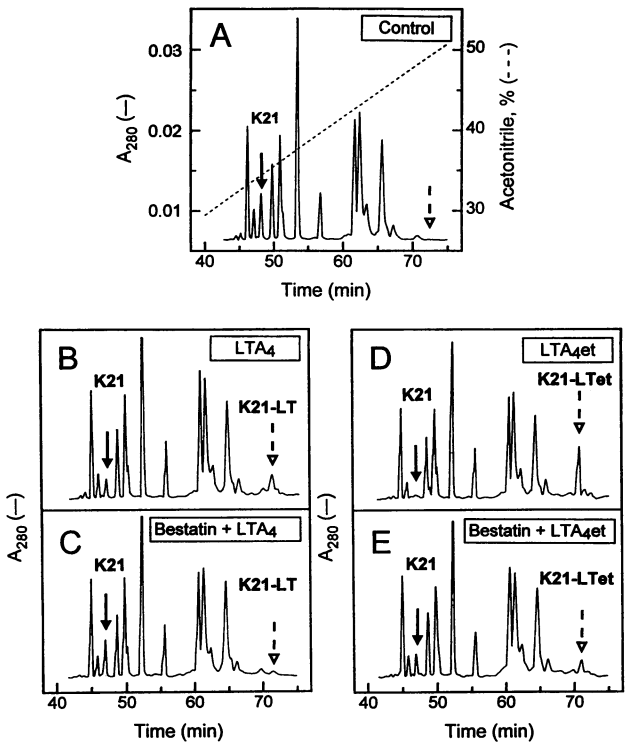

FIG. 4. Protection by bestatin from modification of peptide K21 with leukotriene epoxides. LTA_4 hydrolase (100 μg/ml) was preincubated in the presence or absence of 2 mM bestatin for 60 min prior to addition of 5×13 μM LTA_4 or LTA_4 ethyl ester. The enzyme samples were gel-filtered, carboxymethylated, cleaved with Lys-C, and analyzed by reversed-phase HPLC. Shown are the chromatograms (42–75 min) obtained with the absorbance monitored at 280 nm. (*A*) Profile of peptides generated with a control digest of untreated LTA_4 hydrolase. (*B* and *C*) Corresponding results from cleavages of enzyme treated with LTA_4 in the absence (*B*) or presence (*C*) of bestatin. (*D* and *E*) Patterns observed with enzyme treated with LTA_4 ethyl ester (LTA_4et) in the absence (*D*) or presence (*E*) of bestatin. Solid arrows indicate the position of peptide K21, and open arrows the position of peptides K21-LT (*B* and *C*) or K21-LTet (*D* and *E*).

structural isomers of peptide K21-LT (see below, regarding modification of peptide K21 with LTA_4 ethyl ester).

Suicide Inactivation of LTA_4 Hydrolase by LTA_4 Methyl and Ethyl Esters. Apparent binding constants and inactivation rate constants for the two enzymatic activities of LTA_4 hydrolase during suicide inactivation have been shown to be compatible with a single type of substrate–enzyme complex which partitions between two fates, turnover and inactivation (14). For inactivation by LTA_4, partition ratios (turnover/inactivation) of 129 ± 16 for the epoxide hydrolase and 124 ± 10 for the aminopeptidase activity have been reported, which demonstrates that both catalytic activities are equally affected by this process (15). In the case of LTA_4 methyl ester, no turnover is observed, but inactivation occurs with a rate constant k_{inact} = 1.1 min^{-1} and an apparent binding constant K_I = 3.6 μM. Almost identical values are obtained for the corresponding kinetic constants for unesterified LTA_4 (k_{inact} = 0.8 min^{-1}, K_I = 3.7 μM) (15). Since the concentrations of LTA_4 methyl and ethyl ester are not depleted by any significant enzymatic conversion to the corresponding esters of LTB_4, and esterified LTA_4 is less labile than the free acid, LTA_4 hydrolase can be inactivated more efficiently with the ester derivatives. In our hands, five additions of LTA_4 methyl and ethyl ester (final concentration, 60–65 μM) inactivated LTA_4 hydrolase by >80%. As observed with unesterified LTA_4, the degree of inactivation was reflected by a proportionate loss of peptide K21 in corresponding peptide maps (Table 1). Additionally, for inactivation by LTA_4 methyl and ethyl esters, loss of peptide K21 was accompanied by the appearance of novel, presumably modified, peptides which were eluted at retention times almost identical to that of peptide K21-LT (Fig. 4).

Binding of LTA_4 Ethyl Ester to Tyr-378 of Peptide K21. Suicide inactivation of LTA_4 hydrolase by LTA_4 ethyl ester was accompanied by an almost complete loss of peptide K21 and the appearance of a novel peak with prominent absorbance at 280 nm, denoted K21-LTet. Sufficient amounts of this peptide were obtained to allow complete Edman degradation. Sequence analysis revealed that residues 1–13 and 15–21 of K21-LTet were identical to the sequence of the parent peptide K21. However, at position 14 there was a gap, which corresponds to Tyr-378 of intact LTA_4 hydrolase (Fig. 3). Thus, we concluded that LTA_4 hydrolase had been covalently modified by LTA_4 ethyl ester via Tyr-378 to produce the peptide K21-LTet after cleavage with Lys-C protease.

In addition to peptide K21-LTet, several small unidentified peaks appeared in peptide maps of LTA_4 hydrolase that had been suicide-inactivated with LTA_4 ethyl ester. Since LTA_4 and its esters contain a highly reactive allylic epoxide, indiscriminate attachment to various nucleophilic groups at sites different from the active center cannot be excluded as a cause for inactivation, particularly in experiments using high concentrations of LTA_4 ($>>K_m$ or K_i). In this way, additional peaks could potentially be formed by reactions of LTA_4 with amino acid residues outside peptide K21. However, little modification at secondary site(s) was observed after a single incubation of enzyme with 100 μM LTA_4 (15) or with 400 μM LTA_4 methyl ester (14). In our experiments, the actual concentration of LTA_4 methyl or ethyl ester never exceeded 13 μM per addition and the final concentration after five additions did not reach 70 μM. Consequently, it seems unlikely that the small extra peaks contained adducts with other peptide backbones. Rather, these peaks may represent lipid–protein adducts at other site(s) of peptide K21 or isomers of the lipid and/or peptide part of K21-LTet, generated during sample preparation.

Protection with Bestatin Against Enzyme Inactivation and Modification of Peptide K21. The competitive inhibitors bestatin and captopril protect LTA_4 hydrolase from inhibition and covalent modification during suicide inactivation (15, 16). To demonstrate that covalent binding of LTA_4 and its esters to peptide K21 was an active-site-directed process, LTA_4 hydrolase (2.9 nmol/2 ml) in 50 mM Tris (pH 8) was preincubated in the presence or absence of 2 mM bestatin and treated five times with ≈13 μM LTA_4, LTA_4 methyl ester, or LTA_4 ethyl ester. After removal of bestatin and reversibly bound hydrolysis products of the leukotriene epoxides by gel filtration, the enzyme was subjected to Lys-C digestion and activity determinations. The inactivation of the epoxide hydrolase and peptidase activities of LTA_4 hydrolase, as well as the loss of peptide K21, were largely prevented by preincubation with bestatin (Table 2 and Fig. 4). In addition, the remaining enzyme activities matched perfectly the modification of peptide K21 (Table 2). Some inactivation and peptide modification occurring even in the presence of bestatin could be explained by high affinities (as judged by K_m or K_i) of the leukotriene epoxides for the enzyme, thus competing with the inhibitor, or by incomplete protection of the active site at saturating concentrations of bestatin.

Peptide K21 Contains an Active-Site Residue(s). The results of the present study strongly indicate that peptide K21 contains an active-site residue(s). Thus, the loss of peptide K21 in peptide maps of suicide-inactivated LTA_4 hydrolase, the appearance of a modified more lipophilic peptide K21-LT, and the correlation between reduction of peptide K21 and degree of enzyme inactivation demonstrate that K21 is involved in binding of LTA_4 to the native protein during mechanism-based inactivation. Furthermore, amino acid sequence analysis of

Table 2. Protection with bestatin from inactivation of LTA_4 hydrolase and modification of peptide K21 by LTA_4 and LTA_4 methyl and ethyl esters: Residual enzyme activities and presence of peptide K21

Enzyme treatment	Epoxide hydrolase activity, %	Peptidase activity, %	Amount of peptide K21, %
LTA_4	57	56	50
Bestatin + LTA_4	96	86	108
LTA_4 methyl ester	21	23	23
Bestatin + LTA_4 methyl ester	90	78	89
LTA_4 ethyl ester	15	10	14
Bestatin + LTA_4 ethyl ester	84	68	71

The epoxide hydrolase activity, peptidase activity, and amount of peptide K21 detected in samples of LTA_4 hydrolase treated with leukotriene epoxides in the presence or absence of bestatin are expressed in percent of values obtained with untreated control enzyme.

peptide modified by LTA_4 ethyl ester (K21-LTet) points to the involvement of Tyr-378 in this process. In addition, the protective effects of the competitive inhibitor bestatin on enzyme inactivation and peptide modification corroborate the conclusion that peptide K21 contains structural and functional elements of the active site(s). In agreement with this view, recent data obtained by computer-assisted sequence comparisons and site-directed mutagenesis have identified another tyrosine residue (Tyr-383), also located in peptide K21 (see Table 2), as a potentially catalytic residue involved primarily in peptidolysis (21).

Catalytic Regions in the Structure of LTA_4 Hydrolase. Two regions critically involved in binding or catalysis of LTA_4 and peptide substrates have now been identified. The first, which is defined by the zinc binding signature, includes the ligands of the catalytic zinc ion, His-295, His-299, and Glu-318, as well as Glu-296, which probably acts as a base in peptidolysis (9). This segment was discovered by amino acid sequence comparisons with other zinc hydrolases, all of which are proteases and peptidases (22, 23). Accordingly, mutational analysis has generated information mostly applicable to the peptidase activity of LTA_4 hydrolase and, at present, much less is known about the epoxide hydrolase activity. In fact, with the exception of the zinc atom and its amino acid ligands, no structural elements involved in epoxide hydrolysis have previously been identified. Hence, we believe that the henicosapeptide (K21) described in the present investigation is the first peptide segment in LTA_4 hydrolase that has been directly linked to this enzymatic activity and may thus be regarded as a second catalytic region. Although the borders of this second region are not defined, it appears to be centered around the two tyrosine residues of the henicosapeptide (see Table 2). In this context, it is interesting that recent work employing chemical modification with group-selective reagents and protection with bestatin has demonstrated that two tyrosines appear to be located at the active site of LTA_4 hydrolase (24). Further work with site-directed mutagenesis will clarify the role of Tyr-378 and other residues of peptide K21 in the molecular mechanisms of suicide inactivation and catalyses of LTA_4 hydrolase, information which may prove instrumental for the design of potent and selective enzyme inhibitors.

We express our sincere gratitude to Dr. A.W. Ford-Hutchinson for his generous supply of indispensable materials. This study was supported by funds from the Swedish Medical Research Council (03X-10350, 03X-217, and 13X-3532) and Magnus Bergvall's foundation. M.J.M. is a recipient of a fellowship from the Deutsche Forschungsgemeinschaft.

1. Ford-Hutchinson, A. W. (1990) *Crit. Rev. Immunol.* **10,** 1–12.
2. Arand, M., Grant, D. F., Beetham, J. K., Friedberg, T., Oesch, F. & Hammock, B. D. (1994) *FEBS Lett.* **338,** 251–256.
3. Haeggström, J. Z., Wetterholm, A., Vallee, B. L. & Samuelsson, B. (1990) *Biochem. Biophys. Res. Commun.* **173,** 431–437.
4. Minami, M., Ohishi, N., Mutoh, H., Izumi, T., Bito, H., Wada, H., Seyama, Y., Toh, H. & Shimizu, T. (1990) *Biochem. Biophys. Res. Commun.* **173,** 620–626.
5. Griffin, K. J., Gierse, J., Krivi, G. & Fitzpatrick, F. A. (1992) *Prostaglandins* **44,** 251–257.
6. Orning, L., Gierse, J. K. & Fitzpatrick, F. A. (1994) *J. Biol. Chem.* **269,** 11269–11273.
7. Örning, L., Krivi, G. & Fitzpatrick, F. A. (1991) *J. Biol. Chem.* **266,** 1375–1378.
8. Örning, L., Krivi, G., Bild, G., Gierse, J., Aykent, S. & Fitzpatrick, F. A. (1991) *J. Biol. Chem.* **266,** 16507–16511.
9. Haeggström, J. Z., Wetterholm, A., Medina, J. F. & Samuelsson, B. (1993) *J. Lipid Mediators* **6,** 1–13.
10. McGee, J. & Fitzpatrick, F. (1985) *J. Biol. Chem.* **260,** 12832–12837.
11. Evans, J. F., Nathaniel, D. J., Zamboni, R. J. & Ford-Hutchinson, A. W. (1985) *J. Biol. Chem.* **260,** 10966–10970.
12. Sun, F. F. & McGuire, J. C. (1984) *Biochim. Biophys. Acta* **794,** 56–64.
13. Ohishi, N., Izumi, T., Minami, M., Kitamura, S., Seyama, Y., Ohkawa, S., Terao, S., Yotsumoto, H., Takaku, F. & Shimizu, T. (1987) *J. Biol. Chem.* **262,** 10200–10205.
14. Orning, L., Jones, D. A. & Fitzpatrick, F. A. (1990) *J. Biol. Chem.* **265,** 14911–14916.
15. Orning, L., Gierse, J., Duffin, K., Bild, G., Krivi, G. & Fitzpatrick, F. A. (1992) *J. Biol. Chem.* **267,** 22733–22739.
16. Evans, J. F. & Kargman, S. (1992) *FEBS Lett.* **297,** 139–142.
17. Haeggström, J., Rådmark, O. & Fitzpatrick, F. A. (1985) *Biochim. Biophys. Acta* **835,** 378–384.
18. Wetterholm, A., Medina, J. F., Rådmark, O., Shapiro, R., Haeggström, J. Z., Vallee, B. L. & Samuelsson, B. (1991) *Biochim. Biophys. Acta* **1080,** 96–102.
19. Wetterholm, A., Macchia, L. & Haeggström, J. Z. (1994) *Arch. Biochem. Biophys.* **318,** 263–271.
20. Wetterholm, A. & Haeggström, J. Z. (1992) *Biochim. Biophys. Acta* **1123,** 275–281.
21. Blomster, M., Wetterholm, A., Mueller, M. J. & Haeggström, J. Z. (1995) *Eur. J. Biochem.*, in press.
22. Malfroy, B., Kado-Fong, H., Gros, C., Giros, B., Schwartz, J.-C. & Hellmiss, R. (1989) *Biochem. Biophys. Res. Commun.* **161,** 236–241.
23. Vallee, B. L. & Auld, D. S. (1990) *Biochemistry* **29,** 5647–5659.
24. Mueller, M. J., Samuelsson, B. & Haeggström, J. Z. (1995) *Biochemistry* **34,** 3536–3543.