Chemical Modification of Leukotriene A_4 Hydrolase. Indications for Essential Tyrosyl and Arginyl Residues at the Active Site†

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ABSTRACT: We have employed chemical modification to identify amino acids essential for the catalytic activities of the bifunctional zinc metalloenzyme leukotriene A_4 hydrolase (EC 3.3.2.6). The epoxide hydrolase and the peptidase activity were both rapidly inactivated by *N*-acetylimidazole and tetranitromethane. Furthermore, treatment with 2,3-butanedione and phenylglyoxal also resulted in loss of both activities. Leukotriene A_4 hydrolase could be protected from inactivation by these tyrosyl and arginyl reagents by the competitive inhibitors bestatin and captopril, respectively. Two tyrosyl and three arginyl residues were found by differential labeling techniques to be protected by the inhibitors, which thus suggested that these amino acids are located close to or at the active center of the enzyme. Limited modification by thiol reagents and particularly methyl methanethiosulfonate led to a >10-fold increase in the peptidase activity and a decreased epoxide hydrolase activity, whereas prolonged treatment inhibited both activities. Kinetic analysis of modified enzyme, using the substrate alanine *p*-nitroanilide, revealed that the stimulatory effect on the peptidase activity was due to increased enzyme turnover, whereas the Michaelis constant remained unaffected. Furthermore, the modified enzyme displayed a reduced apparent affinity constant for chloride ions, which strongly stimulate the peptidase activity. Neither activation nor inactivation by methyl methanethiosulfonate was influenced by the presence of competitive inhibitors, which suggested that this compound did not react with amino acids at the active center but rather with residues of importance for the overall enzyme conformation.

Leukotriene (LT)[1] A_4 hydrolase (EC 3.3.2.6) is a bifunctional zinc metalloenzyme (Haeggström et al., 1990; Minami et al., 1990) which catalyzes the hydrolysis of the unstable epoxide intermediate LTA_4 into the proinflammatory substance leukotriene B_4 (LTB_4) and also exhibits an amidase/peptidase activity toward synthetic substrates. Leukotriene B_4 is a potent chemotactic agent for leukocytes and stimulates a number of other leukocyte functions, including aggregation, enhancement of lysosomal enzyme release, superoxide anion production, enhancement of complement-dependent cytotoxicity reactions, and leukocyte adherence to the endothelium (Ford-Hutchinson, 1990). Since there is evidence for increased LTB_4 formation in human inflammatory diseases, this compound has the potential for being an important mediator in a number of disease states (Fretland et al., 1990). The physiological significance of the aminopeptidase activity is largely unclear and remains to be evaluated. The naturally occurring enkephalins are substrates *in vitro* for this activity; however, their hydrolysis is inefficient compared with that of LTA_4 (Griffin et al., 1992). Recent data suggest that LTA_4 hydrolase is an arginine aminopeptidase with high efficiency for several tripeptides (Orning et al., 1994).

The enzyme has been purified to homogeneity from various sources as a monomeric soluble protein (M_W 69 000 Da), and the cDNAs coding for the human, mouse, and rat enzymes have been cloned and sequenced (Funk et al., 1987; Minami et al., 1987; Medina et al. 1991a; Makita et al., 1992). Sequence comparisons with certain zinc metalloenzymes, *e.g.*, thermolysin and aminopeptidase M, revealed a zinc-binding motif in LTA_4 hydrolase. In agreement with the sequence-based predictions, the three zinc-binding ligands were identified as His-295, His-299, and Glu-318 by site-directed mutagenesis and zinc analysis (Medina et al., 1991b). By the same technique, glutamic acid in position 296 of LTA_4 hydrolase, a residue which is conserved in the zinc-binding motif of several zinc proteases and peptidases, was shown to be directly involved in the catalytic mechanism of the peptidase reaction but not in the epoxide hydrolase reaction (Wetterholm et al., 1992). Thus, in analogy with a mechanism suggested for thermolysin, Glu-296 was proposed to participate in the peptidolysis as a general base (Kester & Matthews, 1977; Pangburn & Walsh, 1975). The data collected so far (Haeggström et al., 1993) have shown that the epoxide hydrolase and peptidase activities differ in many respects, *e.g.*, susceptibility to substrate-mediated inactivation, stimulation by monovalent anions, metal inhibition, and dependence on Glu-296, but also share properties such as dependence on the intrinsic zinc atom for catalysis, inhibition by LTA_4, and susceptibility to inhibition by bestatin and captopril, inhibitors of aminopeptidases and angiotensin converting enzyme, respectively. The active sites, defined as the sum of all functional elements participating in the catalytic mechanism(s), thus seem to be at least in part identical.

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[1] Abbreviations: LT, leukotriene; TNM, tetranitromethane; NEM, *N*-ethylmaleimide; MMTS, methyl methanethiosulfonate; TNBS, trinitrobenzenesulfonate.

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In view of the potential importance of LTA_4 hydrolase as a pharmacological target for treatment of inflammatory disorders, a detailed understanding of the catalytic mechanism(s) is required for the design of potent and specific enzyme inhibitors. Although recent studies have unraveled a number of previously unknown properties of the enzyme, our present knowledge about the structure and function of LTA_4 hydrolase is not detailed and is thus in many respects incomplete. Since the three-dimensional structure of LTA_4 hydrolase is not yet known, we initiated studies with chemical modification in order to screen for the amino acids that are of importance for the respective enzyme activities.

EXPERIMENTAL PROCEDURES

Materials. LTA_4 ethyl ester (a generous gift from Dr. A. W. Ford-Hutchinson, Merck-Frosst, Canada) was saponified in tetrahydrofuran with 1 M LiOH (6%, v/v) for 48 h at 4 °C. Alanine *p*-nitroanilide, captopril, bestatin, *N*-acetylimidazole, methyl methanethiosulfonate, iodoacetamide, iodoacetic acid, *N*-ethylmaleimide, 1-chloro-2,4-dinitrobenzene, 2,3-butanedione, phenylglyoxal, 2,4,6-trinitrobenzenesulfonic acid, and sodium cyanoborohydride were from Sigma. Tetranitromethane and diethyl pyrocarbonate were purchased from Aldrich. [7-^{14}C]Phenylglyoxal with a specific activity of 888 Bq/mmol was from Amersham.

Enzyme Purification and Activity Assays. Recombinant human LTA_4 hydrolase, expressed in *Escherichia coli*, was purified to apparent homogeneity, essentially as described (Wetterholm et al., 1991). Purity was established by SDS−PAGE on a Phast system (Pharmacia) using 10−15% gradient gels. Bands of protein were visualized by staining with Coomassie brilliant blue. The epoxide hydrolase activity was determined from short-time (15 s) incubations of enzyme (2−5 μg) in 50 mM Tris-HCl or HEPES (pH 8, 100 μL) with LTA_4 (4−9 nmol) at room temperature. Reactions were quenched with 2 vol of MeOH, PGB_1 was added as internal standard, and samples were extracted and analyzed by RP-HPLC, essentially as described (Wetterholm et al., 1991). The peptidase activity was determined in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, with alanine *p*-nitroanilide (1 mM) as the substrate. The enzymatic product, *p*-nitroaniline, was measured spectrophotometrically at 405 nm in the wells of a microtiter plate, using a multiscan spectrophotometer (MCC/340, Labsystems).

Chemical Modification, General Procedures. Inhibitors were dissolved in a small volume of ethanol and diluted with water. Aliquots of the freshly prepared stock solution were added to the enzyme solution in an appropriate buffer (5 μg of enzyme/100 μL of final solution, 0.72 μM). Reactions were terminated by gel filtration. For acetylations, aliquots from a stock solution of *N*-acetylimidazole in dry toluene were evaporated under a stream of nitrogen and mixed with the enzyme solution. Acetic anhydride, citraconic anhydride, and diethyl pyrocarbonate were diluted in acetonitrile, and 1 μL was added to the vigorously stirred enzyme solution. To perform reductive methylation, LTA_4 hydrolase was incubated with 2 mM formaldehyde and treated with a freshly prepared aqueous solution of sodium cyanoborohydride, 10 mM final concentration, for 1 h.

Experimental conditions for the individual reagents were as follows: 1 h in 50 mM Hepes, pH 7.5, at 25 °C for *N*-acetylimidazole; 15 min in 50 mM Tris-HCl, pH 7.5, at 25 °C for tetranitromethane; 2 h in 50 mM Hepes or borate buffer, pH 8, at 30 °C for 2,3-butanedione; 2 h in 50 mM Hepes, pH 8, at 25 °C for phenylglyoxal; 1 h in 50 mM sodium phosphate buffer, pH 7, at 25 °C for *N*-ethylmaleimide (NEM) and diethyl pyrocarbonate; 1 h in 50 mM Tris-HCl, pH 7.5, at 25 °C for all other thiol reagents; and 10 min in 100 mM Hepes, pH 8, at 25 °C and 1 h in 100 mM Hepes, pH 8.5, at 25 °C for trinitrobenzenesulfonate.

Experiments with Reversible Competitive Inhibitors. Protection from inactivation was generally carried out by preincubating the enzyme for 45 min at 25 °C with 2 mM bestatin or 10 mM captopril, if not stated otherwise in the text, before adding the modifying reagent. The concentrations of the respective inhibitors used in this study are well above the reported K_i values. Thus, at pH 8, captopril inhibited LTA_4 hydrolase with $K_i = 6$ μM for the epoxide hydrolase activity and $K_i = 0.06$ μM for the peptidase activity (Orning et al., 1991a). Corresponding values for bestatin were 0.20 and 0.17 μM (Orning et al., 1991b). Excess reagents and inhibitors were removed by passing the solution through a calibrated gel filtration column (Sephadex G 25 fine, 18 × 1 cm). Protein recovery was checked, in a separate set of experiments, by measuring the peptidase activity of LTA_4 hydrolase before and after gel filtration; 93−105% of the activity was found in the eluate, and therefore an almost complete recovery of protein from the gel filtration column was generally assumed. Enzyme activities were determined 4 h after molecular exclusion chromatography.

Stoichiometry of Enzyme Inactivation by Tetranitromethane and Phenylglyoxal. (A) Modification by Tetranitromethane. LTA_4 hydrolase (276 μg, 4 nmol) in 100 μL of 50 mM Tris-HCl, pH 7.5, was incubated with tetranitromethane in the presence or absence of 2 mM bestatin at 25 °C for 15 min. Gel filtration (column, see above) resulted in baseline separation of reagent and enzyme (peak overlap, 2×10^{-4}%). The degree of nitration was determined on the basis of the absorption of the nitrotyrosyl residue at 381 nm (isosbestic point) using a molar extinction coefficient $\epsilon = 2200$ M^{-1} cm^{-1}, which can be used for quantitation independent of the pH of the solution (Riordan & Vallee, 1972).

(B) Modification by Phenylglyoxal. LTA_4 hydrolase (136 μg, 2 nmol in 100 μL) was incubated in the presence or absence of 10 mM captopril with a mixture of 2×10^6 cpm [7-^{14}C]phenylglyoxal and unlabeled phenylglyoxal (5−37.5 mM final phenylglyoxal concentration). Labeling of the enzyme with ^{14}C was determined by liquid scintillation counting of the radioactivity in protein-containing fractions from size-exclusion chromatography. Phenylglyoxal incorporation was calculated from the specific activity [cpm/nmol of protein] of the modified protein.

RESULTS

Inactivation by N-Acetylimidazole and Tetranitromethane. The time course of inactivation of LTA_4 hydrolase peptidase activity by *N*-acetylimidazole is shown in Figure 1. Treatment with 10 mM *N*-acetylimidazole reduced the peptidase activity to 8% and the epoxide hydrolase activity to 22% of the initial activity within 75 min. Activities did not return after gel filtration, but both activities were restored to about 90% of the initial activity by addition of hydroxylamine (final concentration, 1 M). Treatment of the native enzyme with

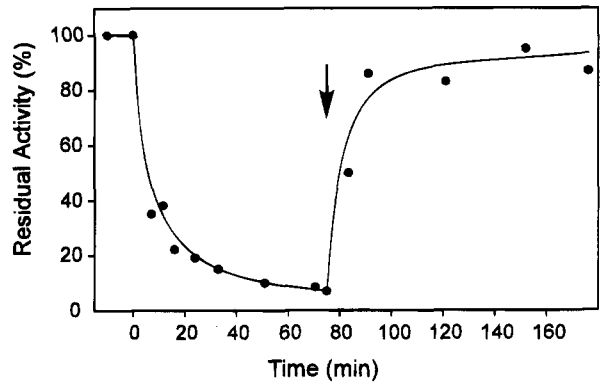

FIGURE 1: Inactivation of the peptidase activity of LTA_4 hydrolase by *N*-acetylimidazole and restoration of the enzymatic activity by 250 mM hydroxylamine. The time of hydroxylamine addition is indicated by an arrow.

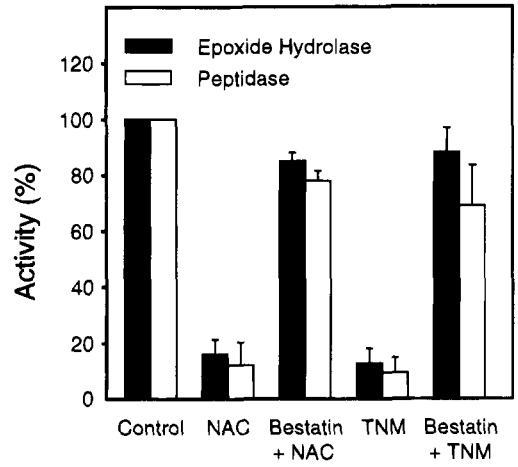

FIGURE 2: Inactivation of LTA_4 hydrolase by 10 mM *N*-acetylimidazole (NAC) for 1 h and by 10 mM tetranitromethane (TNM) for 15 min in the presence or absence of 2 mM bestatin. The relative activities are expressed as mean ± SD of experiments carried out in triplicate.

1 M hydroxylamine for 4 h had no effect on the enzyme activities. Bestatin, a competitive inhibitor of LTA_4 hydrolase, could partially protect the enzyme from acetylation by acetylimidazole. Thus, after treatment for 1 h in the absence or presence of 2 mM bestatin, the peptidase activity decreased to 12 ± 8% and 78 ± 4% (mean ± SD, $n = 3$), respectively. Corresponding values for the epoxide hydrolase activity were 16 ± 5% and 85 ± 3% of the initial activity (Figure 2). Inactivation by acetylimidazole, restoration of the activities by hydroxylamine, and protection from inactivation by bestatin indicated the presence of catalytically important tyrosyl residues in LTA_4 hydrolase.

This conclusion was further supported by the results of modification with tetranitromethane (TNM). Less than 15% of the original activities remained after treatment of 275 μg of enzyme (4 nmol) with 10 mM TNM for 15 min. Inactivation was largely prevented by preincubation of the enzyme with 2 mM bestatin before addition of TNM (final concentration, 10 mM). In a protection experiment carried out in triplicate, 88 ± 8% of the epoxide hydrolase and 70 ± 15% of the peptidase activity (mean ± SD) was recovered after treatment with TNM in the presence of bestatin and subsequent gel filtration, whereas only 13 ± 5% and 9 ± 5% of the initial activities was found after identical treatment of the unprotected enzyme (Figure 2). Tetranitromethane nitrates reactive tyrosine residues to form the colored 3-nitrotyrosine. Under the conditions described above, TNM also oxidizes thiol groups to disulfides and sulfenic and sulfinic acids. Thus, inactivation can be due to modification of either critical tyrosyl or critical cysteinyl residues. Since nitration of tyrosine residues generates a chromophore, the number of nitrated tyrosines can be determined from the absorption spectrum of the modified enzyme after separation from excess reagent and low molecular weight reaction products. In a triplicate experiment using a 250-fold molar excess of TNM over LTA_4 hydrolase (4 nmol/100 μL), only 0.47 ± 0.11 tyrosine was nitrated in the presence of the competitive inhibitor, whereas 2.53 ± 0.08 tyrosines were modified in the unprotected enzyme. Obviously, two reactive tyrosyl residues could be protected by bestatin. Cysteine modification, which possibly also takes place has no influence on this calculation but may contribute to the inactivation of LTA_4 hydrolase. However, LTA_4 hydrolase was only weakly sensitive to modification by other thiol reagents (see below).

Inactivation by 2,3-Butanedione and Phenylglyoxal. 2,3-Butanedione and phenylglyoxal are considered to be highly selective for arginine residues (Means & Feeney, 1971). Treatment of LTA_4 hydrolase with these reagents resulted in a time-dependent loss of enzyme activity. Exposure to 20 mM 2,3-butanedione in 50 mM Hepes, pH 8.0, inactivated 50% of the peptidase and 48% of the epoxide hydrolase activity within 2 h at 30 °C, and essentially no activity could be detected in 50 mM borate, pH 8, under the same conditions. The enhancement of inhibition by borate buffer indicated a 2,3-butanedione-induced arginine modification, due to formation of a borate-stabilized arginine derivative. Rates of inactivation of the epoxide hydrolase and peptidase activities by 2,3-butanedione in borate buffer were measured after gel filtration into 50 mM borate buffer and appeared to obey pseudo-first-order kinetics (Figure 3A,B). The first-order rate constants k_{obs} were plotted as a function of inhibitor concentration, and from the line obtained by linear regression (Figure 3C,D) the second-order rate constants $k_1 = 0.85$ and $k_1 = 0.68$ M^{-1} min^{-1} were calculated for the epoxide hydrolase and peptidase activities, respectively. The constructed line could be extrapolated approximately to the origin, indicating irreversible modification. The ln/ln plots of k_{obs} versus reagent concentration had slopes of 0.67 and 0.70 for the epoxide hydrolase and peptidase activities, respectively, suggesting that one molecule of 2,3-butanedione was needed to inactivate both enzymatic activities of one molecule of enzyme.

The modification was also quite stable to gel filtration into borate-free buffer (50 mM Hepes, pH 8), resulting only in little reactivation of the completely inactivated enzyme due to reversal of the reaction (14 ± 5% and 14 ± 5% of the initial epoxide hydrolase and peptidase activities; *cf.* Figure 4). Furthermore, preincubation with 100 μM bestatin before 2,3-butanedione addition could partially protect the enzyme from inactivation; 60 ± 6% of the epoxide hydrolase activity and 66 ± 8% (mean ± SD, $n = 3$) of the peptidase activity could be recovered after removal of reagent and inhibitor by gel filtration into 50 mM Hepes buffer, pH 8 (Figure 4).

LTA_4 hydrolase was also effectively modified by phenylglyoxal, which reacts with the guanidino group of arginyl residues. The only serious side reaction under the conditions used is the deamination of the α-amino group of the

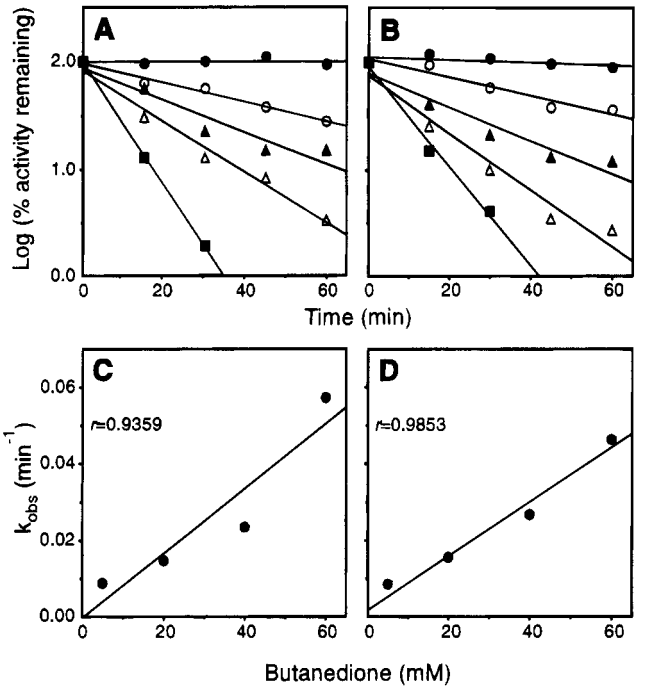

FIGURE 3: Inactivation of LTA_4 hydrolase by 2,3-butanedione in 50 mM borate buffer, pH 8. (A, B) Effect of various concentrations of 2,3-butanedione on the rate of inactivation of the epoxide hydrolase activity (panel A) and peptidase activity (panel B). The enzyme (5 μg) in 50 mM borate buffer, pH 8, was treated at 30 °C with 0 (●), 5 (○), 20 (▲), 40 (△), or 60 mM (■) 2,3-butanedione. The first-order rate constants k_{obs} were determined from the slopes of the straight lines. (C, D) For the epoxide hydrolase (panel C) and peptidase (panel D) activities, the dependence of k_{obs} on [2,3-butanedione] was fitted to a linear equation and from the slope of the lines the second-order rate constants k_1 were calculated.

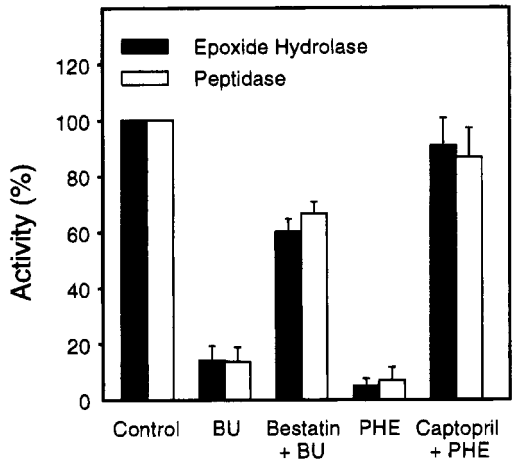

FIGURE 4: Inactivation of LTA_4 hydrolase by 20 mM 2,3-butanedione (BU) in 50 mM borate, pH 8, for 120 min and by 10 mM phenylglyoxal (PHE) for 90 min in 50 mM Hepes, pH 8, in the presence or absence of 100 μM bestatin or 10 mM captopril. The relative activities determined after gel filtration into 50 mM Hepes buffer, pH 8, are expressed as mean ± SD of experiments carried out in triplicate.

N-terminal amino acid to give a residue of the corresponding α-keto acid (Takahashi, 1968).

Plots of the logarithm of remaining activities versus time at different concentrations of phenylglyoxal indicated pseudo-first-order kinetics (Figure 5A,B). Analysis of the dependence of the observed rate constant for enzyme inactivation (k_{obs}) on the phenylglyoxyal concentration suggested that phenylglyoxal bound to the enzyme prior to its reaction. That

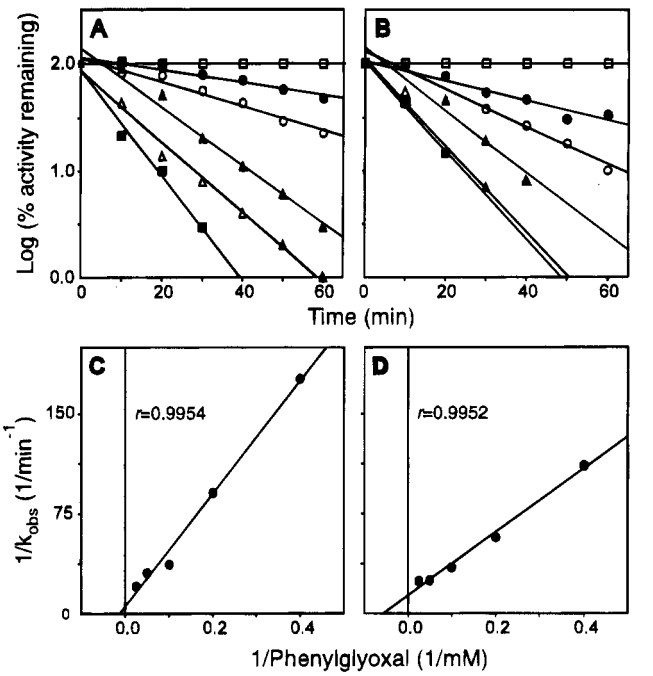

FIGURE 5: Inactivation of LTA_4 hydrolase by phenylglyoxal. (A, B) Effect of various concentrations of phenylglyoxal on the rate of inactivation of the epoxide hydrolase (panel A) and peptidase activities (panel B). The enzyme (5 μg) in 50 mM Hepes, pH 8, was treated at 30 °C with 0 (□), 2.5 (●), 5 (○), 10 (▲), 20 (△), or 40 mM (■) phenylglyoxal. The first-order rate constants k_{obs} were determined from the slopes of the straight lines. From linear plots of $1/k_{obs}$ versus 1/[phenylglyoxal] for the epoxide hydrolase (panel C) and peptidase activities (panel D), dissociation constants and maximal inactivation rates were calculated.

is, plots of k_{obs} versus [phenylglyoxal] were hyperbolic, whereas double-reciprocal plots of the same data conformed well to a linear relationship (Figure 5C,D). Assuming linearity in this latter analysis, it was calculated that phenylglyoxal bound to an arginyl residue critical for the peptidase activity with a dissociation constant of 18 mM and exhibited a maximal inactivation rate of 75 min^{-1} M^{-1}. Corresponding values for the epoxide hydrolase activity were calculated as 81 mM and 192 min^{-1} M^{-1}, for the dissociation constant and the maximal inactivation rate, respectively.

Preincubation of LTA_4 hydrolase with 10 mM of the competitive inhibitor captopril protected the enzyme from inactivation with 10 mM phenylglyoxal for 90 min. The unprotected enzyme lost, under the same conditions, 95 ± 5% of the epoxide hydrolase activity and 91 ± 3% (mean ± SD, $n = 3$) of the peptidase activity (Figure 4).

The stoichiometry of LTA_4 hydrolase inactivation was examined by calculating the incorporation of [7-^{14}C]phenylglyoxal into protected and unprotected enzyme. Modification with a 128-fold molar excess of phenylglyoxal over enzyme (2 nmol/100 μL) resulted in an incorporation of 1.82 mol of phenylglyoxal/mol of enzyme into the unprotected enzyme and no incorporation into the protected enzyme. Since 2 mol of phenylglyoxal combines with 1 mol of arginine (Takahashi, 1968), this corresponds to the modification of 0.91 mol of arginine/mol of unprotected enzyme. Modification of 1 mol of arginine/mol of enzyme inactivated only 45% of the epoxide hydrolase activity and 30% of the peptidase activity. When a higher molar excess of phenylglyoxal was used, both enzyme activities were reduced below 10%. A 500-fold molar excess of reagent resulted in

Table 1: Inactivation of LTA_4 Hydrolase by Thiol-Reactive Reagents[a]

reagent	concn [mM]	% activity [v/vc × 100] epoxide hydrolase	peptidase
none		100	100
Iodoacetamide	10	73 ± 2	83 ± 3
Iodoacetic acid	10	71 ± 4	83 ± 3
MMTS	10	14 ± 1	40 ± 3
MMTS	0.1	43 ± 2	252 ± 14
NEM	10	46 ± 4	15 ± 0.7
NEM	1	82 ± 5	69 ± 7
1-chloro-2,4-dinitrobenzene	10	77 ± 3	88 ± 10
diamide	10	80 ± 15	81 ± 4
diamide/glutathione	10 + 10	5 ± 4	0

[a] For experimental details, see Experimental Procedures.

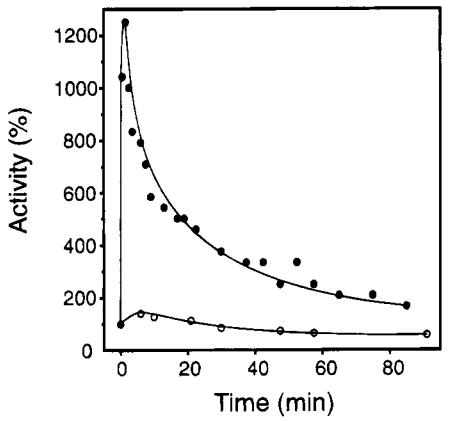

FIGURE 6: Time course for the stimulation of the peptidase activity of LTA_4 hydrolase by 1 mM MMTS (●) or 1 mM NEM (○).

modification of 3.1 mol of Arg/mol of unprotected enzyme and 0.3 mol of Arg/mol of protected enzyme. The experiment was repeated twice, and 3.8 (3.7) mol of Arg/mol of unprotected enzyme and 0.7 (0.5) mol Arg/mol protected enzyme were found to be modified. Finally, when a 1875-fold molar excess of phenylglyoxal was used, 5.2 mol of Arg/mol of unprotected enzyme and 2.08 mol of Arg/mol of protected enzyme were modified. Apparently, three reactive arginines out of a total of 23 arginines present in LTA_4 hydrolase could be protected by captopril.

Effects of Thiol Reagents. A variety of thiol-specific reagents were tested and shown to inactivate LTA_4 hydrolase only moderately, with the exception of the combination of diamide/glutathione (Table 1). Instead, modification of LTA_4 hydrolase with methyl methanethiosulfonate (MMTS) rapidly (within 2 min) increased the peptidase activity more than 10 times, whereas prolonged treatment caused a gradual decrease of activity (Figure 6). The epoxide hydrolase activity decreased slowly with no signs of initial stimulation. A less pronounced, transient stimulation of the peptidase activity was also seen with *N*-ethylmaleimide (NEM) and iodoacetamide. However, neither stimulation nor inhibition of the respective enzyme activities (100 μM MMTS for 5 min or 5 mM NEM for 30 min) could be prevented by preincubation with bestatin (Figure 7).

A pool of modified and permanently activated enzyme was isolated for kinetic analysis by quenching the reaction with MMTS at an early time point. Thus, modification of LTA_4 hydrolase with 1 mM MMTS for 5 min increased V_{max} 3.5-fold from 406 nmol/(mg·min) to 1439 nmol/(mg·min), while the apparent K_m value of 0.47 mM for alanine *p*-nitroanilide

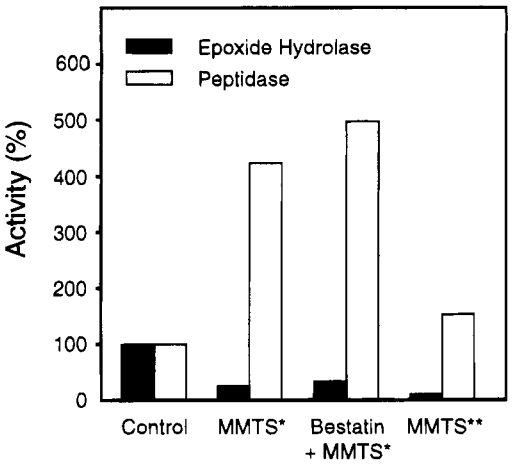

FIGURE 7: Differential stimulation/inhibition of the epoxide hydrolase and peptidase activities of LTA_4 hydrolase by MMTS. The enzyme was treated with 100 μM (*) or 100 mM (**) MMTS for 5 min in the presence or absence of 100 μM bestatin.

was unchanged (Figure 8). Since the hydrolytic activity of LTA_4 hydrolase toward alanine *p*-nitroanilide can be stimulated dose dependently by a number of anions, including Cl^-, we studied the effect of MMTS modification on chloride binding. Apparent affinity constants for native and modified enzyme were calculated as 118 and 37 mM, respectively, demonstrating a higher affinity of the modified enzyme for chloride ions. In addition, MMTS modification increased the optimal reaction velocity achieved by chloride stimulation at a substrate concentration of 1 mM from 508 nmol/(mg·min) of the unmodified enzyme to 1068 nmol/(mg·min) of the modified enzyme, indicating that the higher catalytic efficiency was not due only to the increased affinity for chloride ions (Figure 8). In addition, MMTS-modified enzyme displayed a 5.9-fold higher peptidase activity even in the absence of stimulatory anions (data not shown).

Reagents with Different Specificities. Several reagents with a specificity for lysyl residues have been tested, like acetic anhydride, citraconic anhydride, trinitrobenzenesulfonate (TNBS), and formaldehyde/cyanoborohydride (reductive methylation). These reagents inactivated both activities almost completely except for the reductive methylation, which was without effect (Table 2). However, experiments using captopril and bestatin showed no protection, which may be due to the rapid reaction of these reagents with the thiol or amino group of these inhibitors.

Diethyl pyrocarbonate, which readily reacts with histidines or lysines, did not inhibit the enzymatic activities of LTA_4 hydrolase at a concentration of 5 mM in 50 mM phosphate buffer, pH 7.

DISCUSSION

The recent elucidation of previously unknown structural and functional properties of LTA_4 hydrolase (EC 3.3.2.6) has been initiated through computer-assisted sequence comparisons with other enzymes. For the peptidase activity, it has even been possible to identify a catalytic amino acid and propose a model for the proteolytic mechanism. However, much less is currently known about the epoxide hydrolase activity and the structural elements involved in its mechanism. Both LTA_4 hydrolase and cytosolic epoxide hydrolase (cEH) accept LTA_4 as a substrate, however, the product with the latter enzyme is the vicinal diol (5*S*,6*R*)-

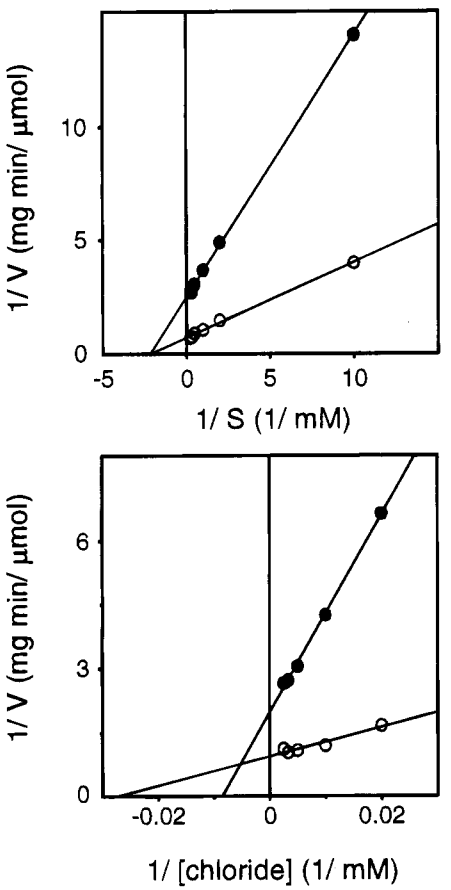

FIGURE 8: Determination of kinetic constants for the peptidase activity of unmodified LTA_4 hydrolase (●) and enzyme modified with 1 mM MMTS for 5 min (○). Samples of enzyme (0.69 µg) were incubated at room temperature for 20 min with different concentrations of alanine *p*-nitroanilide as substrate (0.1−4 mM) in 50 mM Tris-HCl buffer, pH 7.5 (250 µL), containing 100 mM NaCl (upper panel). Apparent affinity constants for chloride were determined by incubating aliquots of the enzyme (0.69 µg) in phosphate buffer (50 mM, pH 7.5, 250 µL) with alanine *p*-nitroanilide (1 mM) at room temperature for 20 min in the presence of 50−400 mM NaCl (lower panel). The specific activities were calculated and the kinetic data were plotted as to Lineweaver−Burk plots. The values are the means of experiments carried out in triplicate.

Table 2: Effect of Various Reagents on LTA_4 Hydrolase Activity[a]

reagent	concn [mM]	% activity [v/vc × 100] epoxide hydrolase	peptidase
none		100	100
citraconic anhydride	10	0	5
acetic anhydride	10	0	0
trinitrobenzenesulfonate	5	2	9
formaldehyde/cyanoborohydride	2 + 10	98	95

[a] For experimental details, see Experimental Procedures.

dihydroxy-*trans*-7,9-*cis*-11,14-eicosatetraenoic acid (5*S*,6*R*-diHETE) instead of LTB_4 (Haeggström et al., 1986). With the recent cloning and sequencing of cEH it became clear that there was no significant sequence homology to LTA_4 hydrolase, and thus no possibility to identify conserved and potentially functional amino acids (Arand et al., 1994).

Covalent modification reagents are useful in screening for amino acid residues which are essential for substrate binding and catalysis. It is particularly useful to have competitive inhibitors for the enzyme, since protection against inactivation by such inhibitors is the usual criterion to assess that modification is active site directed. In the case of LTA_4 hydrolase two competitive inhibitors, captopril and bestatin, are available. Both inhibitors have been shown to prevent substrate (LTA_4)-mediated suicide inactivation and accompanying covalent modification of LTA_4 hydrolase (Evans & Kargman, 1992; Orning et al., 1992), suggesting specific binding to and protection of the overlapping active sites of the enzyme.

The tyrosine reagent *N*-acetylimidazole rapidly inactivated both catalytic activities of LTA_4 hydrolase. Restoration of enzymatic activities by hydroxylamine demonstrated that indeed tyrosyl residues were responsible for the loss of activity, since *N*-acetyllysines, which are occasionally formed, cannot be deacetylated with this reagent (Riordan & Vallee, 1967). Treatment of LTA_4 hydrolase with *N*-acetylimidazole in the absence or presence of bestatin showed that residue(s) critical for both enzymatic activities could be partially protected by the inhibitor. Some inactivation of the enzyme occurred even in the presence of inhibitor. In part, this appears to reflect incomplete protection and/or nonspecific inactivation accompanying extensive acetylation.

In line with these findings were the results obtained with tetranitromethane, which selectively nitrates tyrosine residues. Under the reaction conditions used in our experiments, TNM may oxidize cysteine residues as well (Riordan & Vallee, 1972). Inactivation of both enzymatic activities by TNM could be partially prevented by preincubating the enzyme with bestatin prior to the addition of TNM, indicating the presence of a critical tyrosyl or cysteinyl residue at the active site of the enzyme. Since the enzyme could not be protected from inactivation by the specific and highly reactive cysteine reagents NEM and MMTS, and since other thiol reagents were poor inhibitors, this suggested that inactivation by TNM was mainly due to tyrosine modification at the active site. The incomplete protection of the enzyme may therefore be explained by additional unspecific modifications of cysteinyl or tyrosyl residues.

Measurements of the number of nitrated tyrosines/enzyme molecule by UV revealed that out of the 22 tyrosine residues present in the enzyme, only a few appeared to be reactive. Furthermore, modification in the presence or absence of bestatin (using a 250-fold molar excess of TNM) showed that two tyrosyl residues/enzyme molecule could be protected by the inhibitor, which suggested that these residues are located close to or at the active site(s). In the presence of bestatin, almost no tyrosyl modification could be observed in these experiments with limited modification.

The presence of at least one essential tyrosine at the active center would not be entirely unexpected. Sequence comparison of LTA_4 hydrolase with aminopeptidase N has revealed the presence of a short homologous peptide segment, encompassing six amino acids, which contains a conserved tyrosine residue (Minami et al., 1992; Watt & Yip, 1989). In LTA_4 hydrolase this amino acid would correspond to Tyr-383 and has been suggested as a potential proton donor in the peptidase reaction (Minami et al., 1992). Since both activities were affected by N-acetylimidazole and TNM, a tyrosyl residue might well be involved also in the epoxide hydrolase reaction.

Bestatin and captopril protected LTA_4 hydrolase from inactivation of both of its activities by the arginine-modifying reagents 2,3-butanedione and phenylglyoxal, suggesting the

presence of essential arginyl residues at the active sites. However, the data obtained with the two reagents differ in certain respects. 2,3-Butanedione was shown to react directly with one arginyl residue of the enzyme, resulting in a simultaneous, time-dependent loss of both enzyme activities (Figure 3). On the other hand, studies of the inactivation kinetics using phenylglyoxal revealed that this reagent bound to essential arginyl residues prior to their irreversible modification. The dissociation constants of these complexes as well as the maximal inactivation rates for the epoxide hydrolase and peptidase reactions were different from each other in the same set of experiments (Figure 5). This could be explained by the presence of more than one arginine at the active center such that inactivation of each enzyme activity is related to modification of a separate arginyl residue. Differential labeling experiments, using radiolabeled phenylglyoxal, supported this hypothesis and demonstrated that three arginyl residues could be protected by captopril. In experiments employing different concentrations of phenylglyoxal the three residues were shown to be the most reactive out of the 23 arginines of the enzyme.

Thus, with 2,3-butanedione/borate, inactivation of both enzyme activities occurred after derivatization of only one arginyl residue, whereas at least two different arginyl residues appeared to be responsible for the inactivation by phenylglyoxal. The reason for this discrepancy is not clear but may be related to differences in steric hindrance or conformational changes caused by the respective reagent.

Arginine residues in various zinc metallopeptidases and their role in substrate or inhibitor binding have been demonstrated previously, and arginine residues may also be involved in the binding of a peptide substrate to LTA_4 hydrolase. For instance, 2,3-butanedione was shown to interact with arginyl residues involved in substrate binding in neutral endopeptidase (Jackson & Hersh, 1986) and aminopeptidase N (Helene et al., 1991). Furthermore, X-ray crystallographic data for thermolysin has indicated that Arg-203 can form a hydrogen bond with the carbonyl of the P1′–P2′ amide bond of a peptide in agreement with the endopeptidase nature of this bacterial protease (Kester & Matthews, 1977). However, in the case of our amide substrate, alanine *p*-nitroanilide, the mode of interaction with an arginine in LTA_4 hydrolase is not obvious since this synthetic substrate lacks a P1′-P2′ amide bond as well as a free carboxyl group.

It has been argued that arginyl residues are common anion recognition sites in many, if not all, enzymes utilizing anionic substrates (Riordan et al., 1977). Therefore one or more arginyl residues might well be involved in the binding of the carboxyl group of the natural substrate LTA_4. It is worth noticing that LTA_4 methyl ester cannot be converted to LTB_4 methyl ester, in agreement with a role for a positively charged amino acid in carboxylate recognition. Interestingly, arginine modification appears to be quite selective for particular essential residues. Thus, the modification rate of essential arginine residues in proteins has sometimes proven to be much faster than the rate of modification of arginines in short peptides or modification of the free amino acid (Riordan et al., 1977). Recently, the selective modification of particular arginyl residues in lysozyme, which was used as a model protein, was attributed to intramolecular catalysis by neighboring proton acceptor groups rather than to high surface accessibility, which is otherwise of primary importance for the effect of highly reactive agents (Suckau et al., 1992). Thus, it appears reasonable that the high reactivity of the three arginines in LTA_4 hydrolase may be due to intramolecular salt bridges and hydrogen bonds in the active center.

Chemical modification with highly reactive lysyl reagents like the anhydrides and TNBS led to rapid inactivation of the enzyme. The reported side reactivities of the anhydrides (Atassi & Habeeb, 1972; Means & Feeney, 1971) and of TNBS toward amino acids other than lysine (Fields, 1972) make interpretations difficult unless the particular residues modified can be identified. Unfortunately, reductive methylation, a very mild and specific modification procedure, did not significantly inactivate the enzyme, which may be due to incomplete modification. Lysine methylation does not change the charge of the residues modified and causes no extensive steric perturbation of surrounding amino acids (Means, 1977). Hence, the function of a critical lysine might be only partially impaired by methylation (Shapiro & Riordan, 1983). The role of lysyl residues for catalysis thus remains unclear.

Although histidines have been shown to be essential for LTA_4 hydrolase in that they serve as ligands for the Zn^{2+} ion (Medina et al., 1991), it was impossible to modify any essential histidyl residue with diethyl pyrocarbonate. This was unexpected since aminopeptidase N, which contains a zinc-binding motif structurally similar to that of LTA_4 hydrolase, could well be inactivated under the same conditions (Helene et al., 1991). Therefore, the Zn-binding histidines in LTA_4 hydrolase may not be exposed to the protein surface and/or may be protected by the catalytic Zn^{2+} ion.

The results of this study indicate that the involvement of any of the 11 cysteine residues in the catalytic mechanism is unlikely, since inactivation by highly reactive and specific thiol reagents was poor and could not be prevented by preincubation with a competitive inhibitor. Interestingly, some of the reagents and particularly MMTS increased the peptidase activity at the beginning of the modification process. The increased peptidase activity after a short incubation with MMTS was shown to be due to a drastically increased enzyme turnover (k_{cat}) while Michaelis constant (K_m) was unchanged, indicating that the binding of the substrate alanine *p*-nitroanilide was not affected. Chloride and several other monovalent anions are known to stimulate the peptidase activity by increasing V_{max} without affecting K_m (Wetterholm & Haeggström, 1992), and it could be demonstrated that MMTS-modified enzyme displayed a higher affinity for chloride ions in Tris-HCl buffer. In addition, MMTS-modified enzyme displayed a higher peptidase activity even in the absence of stimulatory chloride ions. Thus, certain thiol reagents may induce small changes in the structure leading to a conformation with an increased affinity for stimulatory chloride ions and a higher catalytic efficiency. Prolonged, more extensive thiol modification inactivated slowly the peptidase as well as the epoxide hydrolase activity, perhaps due to more drastic changes of the tertiary structure.

In summary, the results of this study suggest that tyrosyl and arginyl residues are involved in the enzymatic reactions catalyzed by LTA_4 hydrolase. Two tyrosyl and three arginyl residues can be protected from chemical modification by competitive inhibitors. Sequence analysis of labeled peptides from enzymatic digests and experiments with site-directed

mutagenesis can now be carried out to identify the particular residues involved and eventually contribute to a better understanding of the catalytic mechanism(s).

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