The Catalytic Architecture of Leukotriene C₄ Synthase with Two Arginine Residues

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Leukotriene (LT) C₄ and its metabolites, LTD₄ and LTE₄, are involved in the pathobiology of bronchial asthma. LTC₄ synthase is the nuclear membrane-embedded enzyme responsible for LTC₄ biosynthesis, catalyzing the conjugation of two substrates that have considerably different water solubility; that is, amphiphatic LTA₄ as a derivative of arachidonic acid and a water-soluble glutathione (GSH). A previous crystal structure revealed important details of GSH binding and implied a GSH activating function for Arg-104. In addition, Arg-31 was also proposed to participate in the catalysis based on the putative LTA₄ binding model. In this study enzymatic assay with mutant enzymes demonstrates that Arg-104 is needed for the binding and activation of GSH and that Arg-31 is needed for catalysis probably by activating the epoxide group of LTA₄.

Leukotriene C₄ (LTC₄) and its metabolites, LTD₄ and LTE₄, are collectively called the cysteinyl leukotrienes (cys-LTs). They are generated by certain bone marrow-derived proinflammatory cells, such as mast cells, eosinophils, basophils, and monocyte-derived tissue cells, including macrophages and dendritic cells (1–5). They have been implicated in the pathobiology of human bronchial asthma for their direct effect as bronchoconstrictors (6, 7) and permeability-enhancing mediators (8, 9), their presence in urine and bronchoalveolar fluid during exacerbations (10, 11), and the clinical efficacy of therapeutic agents interfering with the biosynthesis or receptor-mediated action of the cys-LTs (12, 13). Therapeutic intervention has also been shown to be effective in allergic rhinitis, acute and chronic urticaria, and angioedema (14–16), indicating a critical role for the cys-LTs in a broad range of allergic diseases.

The biosynthetic pathway of the cys-LTs begins with cytosolic phospholipase A₂-dependent release of arachidonic acid from the outer nuclear membrane (17) and its subsequent metabolism to 5-hydroperoxyeicosatetraenoic acid and then LTA₄ by 5-lipoxygenase in the presence of the 5-lipoxygenase activating protein (18–20). LTA₄ is then conjugated with reduced glutathione (GSH) to form LTC₄ by means of LTC₄ synthase (LTC₄S) (21). Both 5-lipoxygenase activating protein and LTC₄S are integral membrane proteins of the nuclear membrane and belong to the membrane-associated proteins in eicosanoid and glutathione metabolism superfamily (22, 23). Intracellular LTC₄ is released by the multidrug resistance protein-mediated, energy-dependent pathway. It undergoes extracellular metabolism to LTD₄ by the cleavage of glutamic acid via γ-glutamyl transpeptidase or γ-glutamyl leukotrienease and then to LTE₄ by removal of glycine by dipeptidases (24, 25). The three sequentially generated cys-LTs differ in extracellular stability such that only LTE₄ is readily detected in urine or at a site of inflammation (26, 27). They are also distinct in their affinity for the cloned receptors CysLT₁ and CysLT₂ receptors (28, 29). Studies of mice with targeted disruption of LTC₄S have extended the appreciation of the role of cys-LTs in models of inflammation beyond their smooth muscle activity. LTC₄S knockout mice have a marked reduction in antigen-induced allergic pulmonary inflammation and in bleomycin-induced pulmonary fibrosis (30, 31).

An adenosine diphosphate-reactive purinergic (P2Y₁₂) receptor was recently reported to be required for LTE₄-dependent pulmonary inflammation (32, 33). In addition, a functional receptor for LTE₄-mediated vascular permeability was observed in mice lacking both the classical CysLT₁ and CysLT₂ receptors (34). An LTE₄-reactive receptor has been considered likely because LTE₄ has pathophysiological effects on airway inflammation in asthma, such as mucosal eosinophilia and airway hyperresponsiveness, although LTE₄ has a much lower affinity for the established cys-LT receptors.

The previous crystal structure study provided a detailed view of the GSH binding of LTC₄S (35, 36). The GSH binding site is formed at the interface of two adjacent monomers in the LTC₄S trimer. Nine amino acid residues interact directly with the bound GSH, and almost all of these amino acid residues are conserved in the amino acid sequence alignment in the mem-

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†1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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brane-associated proteins in eicosanoid and glutathione metabolism family. Tyr-93 and Arg-51, which had been proposed to be responsible for this catalysis based on the previous site-directed mutagenesis analysis (37), were included in the nine amino acid residues for GSH binding. Arg-104 is the only amino acid residue interacting with the thiol group and has been proposed to activate the thiol group of GSH (Fig. 1). The details on the catalytic activity of LTC₄ biosynthesis by LTC₄-S remain to be determined. Based on the previous x-ray crystal structure of the LTC₄-S-GSH complex, how GSH is bound and activated was proposed. However, the amino acid residue(s) that is directly involved in the catalysis of LT₄ was elusive without LT₄ complex structure. We hypothesized that there is a certain amino acid residue forming a hydrogen bond with the epoxide oxygen of LT₄, because protonation of the epoxide group of LT₄ has a powerful impact on the reactivity of LT₄. The epoxide group of LT₄ is hydrolyzed readily in acidic solution, whereas it is less reactive in basic solution (38). In fact, to prepare LT₄ from LT₄ methyl ester (LT₄-Me), the hydrolysis of the methyl group of LT₄-Me proceeds without ring opening of the epoxide group in a basic solution, such as 0.25 M NaOH/acetone, as shown in the product insert from the supplier (Cayman Chemical) and the previous literature (39).

We have proposed that the Arg-31 is the amino acid residue interacting with the epoxide oxygen based on the putative LT₄ binding model (Fig. 1) (35). LT₄ binding models based on the binding of the hydrocarbon tail of dodecyl maltoside in the hydrophobic part of the active site have been proposed (35, 36). The proposed position of the epoxide group is a plausible position for the catalysis, although the proposed binding mode of LT₄ is still controversial due to a lack of the structural complex for LTC₄-S and LT₄. Nevertheless, the proposed LT₄ binding mode is consistent with the 55-hydroxyl-6R-glutathionyl product stereochromy by the Sₑ₂ nucleophilic substitution, where the C6 carbon as the electrophile faces the thiol group of GSH at the narrow path and the leaving epoxy oxygen at the other side of C6. In such a case, Arg-31, the side chain reaches the epoxide group in the putative LT₄ model, suggesting a catalytic role for Arg-31 in the reactivity of LT₄. Arg-31 was proposed to make the epoxide group reactive by forming a hydrogen bond with the epoxide oxygen in the carrying out of the catalytic mechanism. An enzymatic assay is needed to confirm the putative functions of these arginine residues suggested by the crystal structure.

Based on the enzyme assay performed on the wild-type (WT) and mutant LTC₄-S, here we report that the two arginine residues Arg-31 and Arg-104 are the catalytic amino acid residues specific for LT₄ and GSH, respectively. The decreased kₐᵣ/Kᵢₗ of the mutant LTC₄-S missing Arg-31 or Arg-104 showed that the arginine residues closely participate in the catalysis. A comparison of the pH dependence of kᵣ and Kᵢₗ between WT LTC₄-S and these mutants showed that Arg-31 has the LT₄ activating, but not binding function of LT₄ and Arg-104 plays a role in both the activating and binding of GSH.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Plasmids for the mutants of human LTC₄-S in which alanine replaced Arg-31 (R31A) or Arg-104 (R104A), respectively, were prepared using a QuikChange mutagenesis XLII kit (Stratagene). A QuikChange lightning mutagenesis kit (Stratagene) was used for preparation of the expression plasmids in which alanine replaced Arg-90 (R90A), Arg-92 (R92A), Arg-99 (R99A), or Arg-113 (R113A) or glutamine replaced Arg-31 (R31Q), Arg-90 (R90Q), Arg-92 (R92Q), Arg-99 (R99Q), Arg-104 (R104Q), or Arg-113 (R113Q) or glutamic acid replaced Arg-31 (R31E) or leucine replaced Arg-31 (R31L). The template was the pESP-3 expression vector (Stratagene) carrying human LTC₄-S with a His₆ tag at its C terminus (35, 40). The primers for the mutation works are shown in supplemental Table S1.

Protein Expression and Purification—The resultant plasmids of each mutant as well as the plasmid for WT LTC₄-S were introduced into Schizosaccharomyces pombe h− leu1-32 using the lithium acetate method (41), and stable clones were established. Expression of WT and mutant LTC₄-S were induced by the depletion of thiamine in the culture media, as the promoter of the plasmid is highly activated by the depletion of thiamine.

The proteins for the enzyme assay were purified as described (35) with an omission of the last PD-10 desalting step. Thus, LTC₄-S was eluted from a Superose-12 column equilibrated with a solution of 20 mM MES-NaOH (pH 6.5), 0.1 mM NaCl, 0.04% (w/v) dodecyl-β-D-maltoside (DDM), 1 mM DTT, 10%(v/v) glycerol, and 5 mM GSH and was concentrated to ~5
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mg/ml and then stored at −80 °C. Concentrations of the purified enzymes were determined based on UV absorption at 280 nm and the milligram extinction coefficients, 1.57 mg−1·cm−1. The purified samples were confirmed to be a single band using SDS-polyacrylamide gel electrophoresis.

The recombinant enzymes for the crystallographic work were additionally applied to a PD-10 column equilibrated with a solution of 20 mM MES-NaOH (pH 6.5), 0.04%(w/v) DDM, and 5 mM GSH. The recombinant enzymes eluted from the PD-10 column were concentrated to 6.0 mg/ml for WT LTC4S, 5.4 mg/ml for the R31A mutant, and 2.9 mg/ml for the R104A mutant.

Crystallography—The crystals of WT LTC4S and the R31A mutant were grown at 20 °C from a mother solution composed of equal amounts of the enzyme solution and the reservoir solution containing 0.1 M MES-NaOH (pH 6.5), 1.6 M ammonium sulfate, and 45 mM GSH. The crystals were grown at 20 °C from a mother solution composed of equal amounts of the enzyme solution and the reservoir solution containing 0.1 M MES-NaOH (pH 6.5), 2.4 M ammonium sulfate, and 45 mM GSH. The crystals were dipped into the harvest solution supplemented with 15%(v/v) ethylene glycol before the x-ray diffraction experiment at a cryogenic temperature using BL26B2/SPring-8 (42). The statistical data for the diffraction data are shown in Table 1.

The crystals of the R104A mutant were grown under conditions that were virtually the same as in the previous case (35). The R104A mutant was mixed with the reservoir solution (28%(v/v) PEG400, 0.1 M MOPS-NaOH (pH 7.0)) in equal amounts and incubated at 4 °C for 2 weeks. The crystals grown were frozen at a cryogenic temperature, and diffraction images were taken with a BL44B2/SPring-8 (43).

The structural comparison between the WT LTC4S and the mutants of R31A and R104A was performed by the difference Fourier method to assess whether the three-dimensional structure of LTC4S suffered damage from the point mutations. The Fourier coefficients and phases were FR31A(F23) or FR31A(C2221) and FR104A(C2221), respectively. FR31A(F23) and FR104A(C2221) are structure factors of the R31A and R104A mutant crystals with the space group F23 and C2221, respectively. FWT(F23) is structure factor of the WT LTC4S crystal in this work, and φWT(F23) is the phase calculated from the refined structure of WT LTC4S. FWT(C2221) is structure factor of the WT LTC4S crystal used in the previous study (PDB ID 2PNO), and φWT(C2221) is the phase calculated from the coordinate of WT LTC4S (PDB ID 2PNO) (35). The computer programs employed were MOSFLM, SCALA, and TRUNCATE for the processing of diffraction images, AMORE and MOLREP for molecular replacement, REFMAC5 and COOT for the structural refinement (44), and PyMOL for the structural inspection and preparation of figures for structural representation.

Far Ultraviolet Circular Dichroic Analysis—Far ultraviolet circular dichroic (CD) spectra of WT LTC4S and the R31A and R104A mutants at 4.0 °C were measured with a J-715 spectropolarimeter (Jasco). The purified enzymes were applied to a PD-10 column equilibrated with a solution of 2.5 mM MES-NaOH (pH 6.5), 0.04% (w/v) DDM.

Relative Enzyme Activities of Arginine Mutants in Comparison to the WT LTC4S—The effects of point mutation on one of the Arg residues around the entrance of GSH binding site were assessed by the determination of the relative activity of the Arg mutants in comparison with that of WT LTC4S. The conditions for this enzyme assay were 20 ng of enzyme in 200 μl of the solution (10 mM GSH, 21.2 μM LTA4-Me, or 20.0 μM LTA4, 50 mM BisTris propane (pH 7.0), 10 mM MgCl2, 0.015% DDM) at room temperature and an incubation time of 2 min. To terminate the enzyme reaction, 608 μl of a solvent of methanol:acetic acid (75:1 by volume) containing prostaglandin B2 (PGB2) as the internal standard for reverse phase HPLC (RP-HPLC) assay was added. One hundred μl of the final 808-μl solution was applied to the RP-HPLC analysis. The assays for each condition were repeated at least three times.

The LTA4-Me purchased from Cayman Chemical was dried under N2 stream and then solved by ethanol with 3%(v/v) triethylamine, and the ethanol solution was used for the assay. LTA4 was prepared from LTA4-Me by the method described in the product insert (Cayman Chemical). The concentrations of LTA4-Me or LTA4 were determined by UV absorbance at 280 nm (the molar extinction coefficients (ε) = 49,000, as shown in the product insert).

Determination of Kinetic Parameters—To determine the kinetic parameters of WT LTC4S and the R31A and R104A mutants, the enzyme activities were measured with varying concentrations of GSH at pH 7.0, 8.0, and 9.0 at room temperature. Enzyme catalysis was started by the addition of 2 μl of LTA4-Me (1.9–9.2 μM) to 198 μl of solution containing 50 mM BisTris propane (pH 7.0, 8.0, 9.0), 10 mM MgCl2, 9.85–0.05 mM GSH for WT LTC4S, 3.11–0.05 mM GSH for R31A, 49.1–0.05 mM GSH for R104A, 0.015% (w/v) DDM, and the enzyme. The enzyme amounts and reaction times were optimized to detect LTA4-Me by RP-HPLC; WT, 20 ng of enzyme and 1 min reaction; R31A, 600 ng and 10 min; R104A, 200 ng and 8 min. The enzyme assay was terminated as described above.

Assays with varying concentrations of LTA4-Me were also performed for WT LTC4S and the R31A and R104A mutants. The reactions were initiated by a 2-μl aliquot of LTA4-Me to 198 μl of solution of 50 mM BisTris propane (pH 7.0, 8.0, 9.0), 10 mM MgCl2, 10 mM GSH, 0.015% (w/v) DDM, and an appropriate amount of enzyme (WT LTC4S, 20 ng; R31A and R104A mutants, 600 ng). The final concentrations of LTA4-Me were 21.2–0.5 μM for WT LTC4S and 10.6–0.1 μM for the R31A and R104A mutants. The reaction times for WT LTC4S and the R31A and R104A mutants were 2, 8, and 8 min, respectively. The enzyme assays was terminated as described above. The enzyme assay using LTA4 was performed to clarify whether the terminal carboxyl group of LTA4, which is esterified to be a methyl ester in LTA4-Me, exerted an affect on the specificity constant.

The specificity constants (kcat/Km) of the WT LTC4S at pH 7.0 for GSH with a fixed LTA4 concentration (20 μM) and for LTA4 with a fixed GSH concentration (10 mM) were measured following the method described above. The concentration of GSH was varied from 2 to 10 mM for the kcat/Km of GSH, and the concentration of LTA4 was changed from 1 to 20 μM for the kcat/Km of LTA4. The reaction time of this assay was 2 min.
Quantification of LTC₄-Me or LTC₄ in a 100-μl aliquot of the 808-μl terminated sample was carried out by RP-HPLC, as described below. The assays for each condition were repeated at least three times. GraphPad Prism5.0a was used for the calculation of the kinetic parameters by non-linear regression analysis with the Michaelis-Menten equation.

**Reverse Phase HPLC Analysis**—The quantification of LTC₄-Me was performed using RP-HPLC with a SYSTEM GOLD 126 solvent module, a 168 detector, a 508 autosampler (Beckman-Coulter) (37), and a YMC-Pack PolymerC18 (4.6-× 250-mm, S-6 μm). The column was equilibrated with solvent A at a flow rate of 1 ml/min. A mixture of 80 ml of methanol, 120 ml of acetonitrile, and 1.6 ml of acetic acid was diluted to 1 liter by water, and then the pH of the solution was adjusted to pH 6.0 by small aliquots of ethanolamine to prepare solvent A. Solvent B for the RP-HPLC analysis was 100% methanol. The mobile phase for the assay with LTA₄-Me and that for the assay with LTA₄ were 61 and 48% solvent B, respectively, and it was main-

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**TABLE 1**

**Statistics of diffraction data**

| Cell dimensions | a = b = c = 168.0 Å |
|-----------------|--------------------|
| Space group     | F23                |
| Beam Line       | BL26B2/SPRING-8    |

**Resolution (Å)**

| Over all | Inner Shell | Outer Shell |
|----------|-------------|-------------|
| 19.8     | 19.8        | 2.0         |

| Number of observations | Total | Unique |
|-----------------------|-------|--------|
| 305,547               | 30,950| 4.7    |
| 25,689                | 1,005 | 9.7    |
| 34,698                | 4,517 | 2.4    |

| Completeness (%) | Multiplicity |
|------------------|--------------|
| 99.9             | 9.9          |
| 97.3             | 25.6         |

**Refinement**

| Bond lengths (Å) | 0.013 |
| Bond angles (°)  | 1.174 |

**Resolution (Å)**

| R / Rfree | 0.179 / 0.198 (0.225 / 0.246) |

| r.m.s. deviations |
|-------------------|
| Bond lengths (Å)  | 0.013 |
| Bond angles (°)   | 1.174 |

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**R31A**

| Cell dimensions | a = b = c = 168.1 Å |
|-----------------|--------------------|
| Space group     | F23                |
| Beam Line       | BL26B2/SPRING-8    |

**Resolution (Å)**

| Over all | Inner Shell | Outer Shell |
|----------|-------------|-------------|
| 38.6     | 38.6        | 2.9         |

| Number of observations | Total | Unique |
|-----------------------|-------|--------|
| 240,758               | 10,946| 6.2    |
| 6,735                 | 371   | 6.8    |
| 36,198                | 1,592 | 3.2    |

| Completeness (%) | Multiplicity |
|------------------|--------------|
| 100.0            | 22.0         |
| 99.2             | 18.2         |
| 100.0            | 22.7         |

**R104A**

| Cell dimensions | a = 117.4 Å, b = 296.2 Å, c = 207.2 Å |
|-----------------|---------------------------------------|
| Space group     | C222₁                                 |
| Beam Line       | BL44B2/SPRING-8                        |

**Resolution (Å)**

| Over all | Inner Shell | Outer Shell |
|----------|-------------|-------------|
| 23.3     | 23.3        | 4.4         |

| Number of observations | Total | Unique |
|-----------------------|-------|--------|
| 191,227               | 26,590| 4.2    |
| 3,240                 | 724   | 11.4   |
| 28,597                | 3,868 | 1.8    |

| Completeness (%) | Multiplicity |
|------------------|--------------|
| 99.3             | 7.2          |
| 79.5             | 4.5          |
| 100.0            | 7.4          |

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*a* The numbers in the parentheses are R and R<sub>free</sub> at the high resolution shell (1.95-1.90Å).
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Results

Relative Activities of the Arg Mutants of LTC₄S in Comparison to WT LTC₄S—The arginine residues around the entrance of the active site, i.e. Arg-31, Arg-90, Arg-92, Arg-99, Arg-104, and Arg-113, were each subjected to point mutations to evaluate the enzyme activity in comparison to WT LTC₄S. The enzyme activities of the mutant enzymes with LTA₄ and LTA₄-Me were normalized to the enzyme activities of the WT LTC₄S with LTA₄ and LTA₄-Me, respectively. The open and the closed bars depict the relative enzyme activities measured using LTA₄ (F) and LTA₄-Me (M), respectively. The arginine residues mutated in this work are shown by stick models. The ribbon model with green is a monomer in the LTC₄S trimer, and the other two are shown in gray. The translucent stick model with yellow carbons represents the putative LTA₄ binding model, and the alkyl chain of the dodecyl maltoside used for the modeling of the LTA₄ is shown by the translucent stick model.

The enzyme activities of R31A, R104A, and R104Q for LTA₄ were 0.06 ± 0.002, 0.06 ± 0.001, 0.02 ± 0.006, and 0.02 ± 0.001, respectively. The relative enzyme activity of R90A for LTA₄-Me was 0.21 ± 0.03. The enzyme activities of R31A, R104A, and R104Q for LTA₄-Me were lower than the detection limit of the RP-HPLC, therefore, the relative enzyme activities were shown by 0.0 in Fig. 2A. Arg-31 and Arg-104 are the amino acid residues proposed to be the catalytic amino acid residues in a previous report (35, 36). Arg-90 forms a hydrogen bond with the side chain of Asn-57, which is one of the nine amino acid residues that directly binds to GSH (35). Thus, Arg-90 mutations may affect the GSH binding indirectly. Further studies will be needed to elucidate the role of R90 in the catalysis.

We further assessed the enzyme activities of mutant enzymes in which Arg-31 was replaced by a glutamic acid residue (R31E) or a leucine residue (R31L), because R31Q exhibited substantial activity even though the enzyme activity of the R31Q mutant was reduced significantly compared with WT LTC₄S (p < 0.001). The relative enzyme activities of R31Q, R31E, and R31L using LTA₄ as the substrate were 0.32 ± 0.003, 0.3 ± 0.001, and 0.3 ± 0.001, respectively, and all of these results are statistically significant in comparison to WT LTC₄S (p < 0.001).

The enzyme activities of all of the Arg-104 mutants were obviously decreased. The mutants of Arg-31, the side chain of which is hydrophobic or negatively charged, exhibited reduced enzyme activities, whereas R31Q, the side chain of which is a neutral hydrophilic one, maintained substantial enzyme activity. The suppression effect of R31 mutations was profound for both LTA₄ and LTA₄-Me, although the effect for LTA₄ was slightly more modest. Thus, the modification of the terminal carboxyl group of LTA₄ may have some effect; however, overall the enzyme activities were substantially reduced particularly for R31 mutants.

Enzyme Kinetics Analysis of WT LTC₄S, R31A, and R104A Mutants—Because the R31A and R104A mutants displayed the most marked decrease in enzyme activity, we further assessed
their enzymatic kinetic parameters. The $k_{\text{cat}}/K_m$ of WT LTC₄S determined under varying GSH or LTA₄-Me concentrations were significantly higher than those of the R31A and R104A mutants ($p < 0.001$) (Fig. 3, A and B). All of the enzymes exhibited a higher $k_{\text{cat}}/K_m$ at pH 8.0 than at pH 7.0 or 9.0. The $k_{\text{cat}}/K_m$ for GSH of WT LTC₄S at pH 8.0 was $11.90 \pm 1.10 \text{ mm}^{-1}\text{s}^{-1}$, and it was 26- and 770-fold greater than those of the R31A (0.46 ± 0.03 mm$^{-1}$s$^{-1}$) and R104A (0.93 ± 0.06 mm$^{-1}$min$^{-1}$) mutants, respectively. The $k_{\text{cat}}/K_m$ for LTA₄-Me of WT LTC₄S at pH 8.0 was 577 ± 35 μM$^{-1}$s$^{-1}$, and it was 160-fold and 60-fold that of the R31A (3.63 ± 0.09 μM$^{-1}$s$^{-1}$) and R104A (9.70 ± 0.64 μM$^{-1}$s$^{-1}$) mutants, respectively.

The $k_{\text{cat}}/K_m$ of WT LTC₄S activity for LTA₄ or LTA₄-Me as well as GSH at pH 7.0 were measured to examine the kinetic effect of the carboxyl esterification of LTA₄. The $k_{\text{cat}}/K_m$ for LTA₄ and LTA₄-Me of WT LTC₄S at pH 7.0 were 629 ± 6 and 421 ± 52 μM$^{-1}$s$^{-1}$, respectively. The $k_{\text{cat}}/K_m$ for GSH of WT LTC₄S using a fixed concentration of LTA₄ and LTA₄-Me at pH 7.0 were 2.8 ± 0.1 and 4.1 ± 0.3 mm$^{-1}$s$^{-1}$, respectively. The measured $k_{\text{cat}}/K_m$ of WT LTC₄S with LTA₄ and LTA₄-Me were comparable.

Only the R104A mutant exhibited a significant increase of the catalytic constant ($k_{\text{cat}}$) between pH 7.0 and 8.0 ($p < 0.001$) (Fig. 3E). The $k_{\text{cat}}$ of R104A increased from $4.15 \pm 0.80 \text{ min}^{-1}$ at pH 7.0 to $14.01 \pm 0.18 \text{ min}^{-1}$ at pH 9.0 and $11.79 \pm 0.80 \text{ min}^{-1}$ at pH 8.0 with the various GSH concentrations (the solid line in Fig. 3E) and from $1.18 \pm 0.05 \text{ min}^{-1}$ at pH 7.0 to $4.09 \pm 0.44 \text{ min}^{-1}$ at pH 8.0, then $3.19 \pm 0.04 \text{ min}^{-1}$ at pH 9.0 with the variable LTA₄-Me concentration (the dashed line in Fig. 3E). WT LTC₄S maintained a higher level of $k_{\text{cat}}$ over the pH range measured; $1.69 \pm 0.16 \text{ s}^{-1}$ at pH 7.0, $1.97 \pm 0.14 \text{ s}^{-1}$ at pH 8.0 and $2.10 \pm 0.13 \text{ s}^{-1}$ at pH 9.0 for the varied LTA₄-Me (the dashed line in Fig. 3C), $2.11 \pm 0.08 \text{ s}^{-1}$ at pH 7.0, $2.22 \pm 0.12 \text{ s}^{-1}$ at pH 8.0, and $1.72 \pm 0.08 \text{ s}^{-1}$ at pH 9.0 for the varied GSH with a fixed LTA₄-Me concentration (the solid line in Fig. 3C). The R31A mutant maintained a $k_{\text{cat}}$ from pH 7.0 to 9.0; the $k_{\text{cat}}$ from the various GSH concentrations with a fixed LTA₄-Me concentration were $1.46 \pm 0.02 \text{ min}^{-1}$ at pH 7.0, $1.48 \pm 0.04 \text{ min}^{-1}$ at pH 8.0, and $1.33 \pm 0.04 \text{ min}^{-1}$ at pH 9.0 (the solid line in Fig. 3D); those from the various LTA₄-Me concentrations were $1.28 \pm 0.21 \text{ min}^{-1}$ at pH 7.0, $1.21 \pm 0.11 \text{ min}^{-1}$ at pH 8.0, and $1.66 \pm 0.14 \text{ min}^{-1}$ at pH 9.0 (the dashed line in Fig. 3D).

The R104A mutant had a Michaelis constant ($K_m$) for GSH higher than 10 mM over the pH range measured, in contrast to WT LTC₄S and the R31A mutant, with the $K_m$ for GSH at the submillimolar level (Table 2). There is a difference in the pH...
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TABLE 2
Michaelis constants for GSH or LTA₄-Me

|                | Wild type | R31A | R104A |
|----------------|-----------|------|-------|
|                | pH 7.0    | pH 8.0 | pH 9.0 | pH 7.0 | pH 8.0 | pH 9.0 |
| $K_m$           | 0.520 ± 0.032 | 0.188 ± 0.027* | 0.189 ± 0.017* | 0.104 ± 0.004 | 0.0541 ± 0.0053* | 0.0669 ± 0.0078* |
| $K_{LTC₄S}$    | 4.08 ± 0.90  | 3.44 ± 0.43 | 4.45 ± 0.65 | 6.09 ± 1.62  | 5.55 ± 0.62  | 8.39 ± 1.84 |

* $p < 0.001$ as compared to pH 7.0.
  † $p < 0.05$ as compared to pH 7.0.
  ‡ $p < 0.01$ as compared to pH 7.0.

dependence of $K_m$ for GSH between the enzymes with and without Arg-104. The $K_m$ for GSH of the WT LTC₄S at pH 7.0 was significantly higher than at pH 8.0 or 9.0. The pH dependence of the $K_m$ for the WT LTC₄S is comparable with that of the R31A mutant but not the R104A mutant. The R104A mutant had a higher $K_m$ for GSH at pH 9.0 than pH 7.0. All enzymes, including the R104A mutant, had a comparable $K_m$ for LTA₄-Me, although only the R104A mutant exhibited a pH dependence of $K_m$ for LTA₄-Me.

Structural Verification of the R31A and R104A Mutants—To elucidate the role of an individual amino acid residue, it is essential that the mutant enzyme does not suffer damage from the point mutation. Using the crystallographically isomorphous crystals of a mutant enzyme and the WT LTC₄S, the difference Fourier method makes it possible to clarify whether there are any differences between the structures of a given mutant enzyme and the WT LTC₄S.

We obtained crystallographically isomorphous crystals of the R31A mutant and the WT LTC₄S with the space group F23 and a crystal of the R104A mutant isomorphous with the crystal of WT LTC₄S with the space group C222₁, as described previously (35). The crystallographic parameters and the statistics of the diffraction data from those crystals are shown in Table 1.

The crystal structure of WT LTC₄S at 1.9 Å resolution with the space group F23 was refined successfully. The crystallographic $R$ and $R_{free}$ values were 0.179 and 0.198 for the diffraction data and 0.225 and 0.246 for the highest resolution shell of 1.95 to 1.90 Å, respectively (Table 1). There was no amino acid residue with the disallowed main chain dihedral angle of $\phi$ or $\psi$ on the Ramachandran plot.

The overall structure of LTC₄S presented here is basically identical to those previously reported (Fig. 4) (35, 36). In a superimposition between the current and previous structures, the root mean square deviations of the corresponding Ca atoms, except for those from the fifth $\alpha$-helix extruding into the bulk solvent, are less than 0.4 Å. The architecture for GSH binding is conserved (Fig. 4B). The GSH binding site is a V-shaped cleft at each intermonomer interface in the LTC₄S trimer, and nine amino acid residues directly participate in the GSH binding. The minor differences between the current and previous structures are the side-chain conformations of Arg-30 and Arg-104. In the current structure, as shown by the model with the green carbons in Fig. 4B, Arg-30 multiply binds the carboxyl group of the $\gamma$-glutamyl moiety, and Arg-104 interacts with both the thiol group and the carbonyl group of the cysteinyl moiety of GSH. The current side chain conformations of Arg-30 and Arg-104 are similar to those observed in the crystal grown with ammonium sulfate rather than polyethylene glycol as the precipitant agent (35, 36). The side chain of Arg-31 was flexible in the crystal structure, and the electron density corresponding to the side chain before the $Cβ$ of Arg-31 was not apparent in the electron density map contoured at 1.2σ. This refined crystal structure of WT LTC₄S allowed us to visualize the structural differences between WT LTC₄S and the mutants of R31A by the difference Fourier method.

The R104A mutant was crystallized using the conditions as previously reported (35). Under the current crystallization conditions with ammonium sulfate and magnesium chloride as the precipitant agent, WT LTC₄S, in which the guanidino side chain of Arg-104 binds a sulfate ion, crystallized in the space group F23, but the R104A mutant missing the side chain of Arg-104 did not crystallize. Thus, we used the previous crystallization conditions with polyethylene glycol 400 as the precipitant agent for crystallization of the R104A mutant. The R104A mutant crystallized in the space group C222₁.

There were no significant residual electron densities, except for the residual electron density corresponding to the side chain of the mutated amino acid residue in both of the difference Fourier electron density maps for each pair of the isomorphous crystals (Fig. 5). The difference Fourier electron density maps supported the conclusion that the three-dimensional structures of the R31A and R104A mutants did not suffer any damage from the point mutations.

The far-ultraviolet CD analysis showed that the secondary structure composition of WT LTC₄S and the R31A and R104A mutants in solution were not distinguishable from each other. The percentages of the secondary structure of WT LTC₄S,
DISCUSSION

Based on the previous x-ray crystallographic studies, we proposed that Arg-31 and Arg-104 constitute the catalytic architecture for conjugating LTA₄ and GSH in the active site of LTC₄S (Fig. 1) (35). To investigate further, we prepared mutant enzymes and performed the assays for enzymatic analysis to obtain first order kinetics as well as verification of the three-dimensional structures of the mutants.

Role of Arg-31 and Arg-104 in the Catalysis—The \( k_{\text{cat}}/K_m \) of the WT LTC₄S measured at pH 7.0, 8.0, and 9.0 were significantly higher than both of the R31A and R104A mutants (Fig. 3, A and B). The effects of each point mutation on the three-dimensional structures of the mutants were examined by the crystallographic and CD analyses. Using each corresponding isomorphous crystal, there were no indications of any significant structural changes in the difference Fourier maps between each mutant and the WT LTC₄S other than the mutated residue, Arg-31 or Arg-104 (Fig. 5). In the CD analyses, the secondary structural elements of these mutants were similar to those of WT LTC₄S. These results support the conclusion that the decreased \( k_{\text{cat}}/K_m \) of the mutants in comparison with the WT LTC₄S was caused by the lack of the side chain of Arg-31 or Arg-104, not by denaturing or partial unfolding imposed by the point mutation. It cannot be excluded that a certain minor structural perturbation may occur that is undetectable by the CD and the crystallographic analyses. As discussed below, Arg-31 and Arg-104 may play distinct roles in the enzymatic catalysis and cooperate to propagate conjugation between GSH and LTA₄ at physiological pH levels.

The binding function of Arg-104 for GSH was established because the R104A mutant exhibited a higher \( K_m \) for GSH than the WT LTC₄S and R31A mutants, in which the \( K_m \) values are at the submillimolar level (Table 2). Furthermore, the pH dependence of the \( K_m \) for the GSH of the WT LTC₄S, R31A, and R104A mutants supports the binding function of Arg-104, as discussed below. The \( K_m \) for the GSH of the WT LTC₄S and R31A mutant was significantly decreased at pH 8.0 and higher pH (\( p < 0.001 \) against pH 7.0). The \( K_m \) for the GSH of the R104A mutant increases at pH 9.0 (\( p < 0.001 \)), with a decrease at pH 8.0 (\( p < 0.05 \)), in comparison with that at pH 7.0. The decreased \( K_m \) for GSH at alkaline pH indicates that the enzymes with Arg-104 retained a higher affinity for GSH at the alkaline pH measured, in contrast to that of the enzyme without Arg-104, having a lower affinity for GSH at pH 9.0. The positively charged guanidino side chain of Arg-104 interacts with the thiol group of GSH directly in the crystal structure. The thiol group of GSH becomes a negatively charged thiolate anion at alkaline pH. Thus, attractive interaction between the positively charged side chain of Arg-104 and the negatively charged thiolate anion of GSH at alkaline pH contributes to the maintenance of the high affinity for GSH at alkaline pH.

In addition to the binding function, the pH-dependent increase of the \( k_{\text{cat}} \) of the R104A mutant to approximately the \( pK_a \) of the thiol group shows the GSH activating function of Arg-104 at physiological pH (Fig. 3). The value of \( k_{\text{cat}} \) is correlated to deprotonation of the thiol group, resulting in the formation of the thiolate anion as the active species in the catalysis. WT LTC₄S has \( k_{\text{cat}} \) values which are just as high at pH 7.0 as at a pH of 8.0 or higher. Therefore, the thiol group in the active site of WT LTC₄S is considered to be deprotonated, even at pH 7.0. In contrast, the \( k_{\text{cat}} \) of the R104A mutant increases in a pH-dependent manner. The \( k_{\text{cat}} \) of the R104A mutant at pH 8.0 and pH 9.0 is ~3-fold higher than at pH 7.0. Thus, the thiolate anion in the active site missing the Arg-104 increases at pH values higher than pH 7.0. The pH dependence profile of the deprotonation of GSH in the active site of the R104A mutant is concluded to be similar to that of free GSH with a normal \( pK_a \) (49). Thus, the guanidino side chain interacting with the thiol group of GSH is responsible for the activation of the thiol group at pH 7.0 as well as the binding function of GSH, as discussed above.

We have proposed that the guanidino side chain of Arg-31 forms a hydrogen bond with the epoxide oxygen, resulting in the formation of an electron deficient epoxide carbon as the target of nucleophilic attack by the thiolate anion of GSH at the initiation of catalysis (Fig. 1) (35). This is because the protonation of the epoxide group of LTA₄ has a significant impact on the reactivity of the epoxide group (38, 39). Although the guanidino side chain of arginine has an extremely high \( pK_a \), the guanidino side chain can form a hydrogen bond with various polar functional groups, such as hydroxyl groups so forth (50, 51). Furthermore, recent computational studies have proposed that the guanidino side chain of arginine residue can act as a general acid (52, 53). We have also proposed that Arg-31 neutralizes a negative charge growing on the epoxide oxygen in the propagation of the nucleophilic attack on the electro-deficient carbon by the thiol group of GSH (Fig. 1) (35).

The enzymatic assay results for the R31A mutant were consistent with the previous proposal for the roles of Arg-31 in the catalysis (35). The remarkably decreased \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) value...
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of the R31A mutant in comparison to that of the WT LTC₄S suggested that the side chain of Arg-31 interacts with a catalytically essential functional group of LTA₄-Me (Fig. 3). The pH dependence of $k_{cat}$ of the R31A mutant, which was similar with that of the WT-LTC₄S, showed that the side chain of Arg-31 may interact with the functional group of LTA₄-Me, having a pKₐ outside of the measured pH range. The epoxide group of LTA₄ is the essential functional group and also has a pKₐ outside of the measured pH range. This supports Arg-31 being the amino acid residue that interacts with the epoxide group of LTA₄.

Arg-31, but not bulk water, is supposed to form an effective hydrogen bond with the epoxide oxygen, accelerating the enzymatic catalysis. The R31A mutant with Ala-31, having a small apolar side chain, the R31L mutant with Leu-31, with a large apolar side chain, and the R31E mutant with Glu-31, having a negatively charged polar side chain, instead of Arg-31, with the longest and positively charged polar guanidino side chain, exhibited enzyme activity reduced to less than one tenth of the WT LTC₄S (Fig. 2A). In contrast, the R31Q mutant, exhibiting 30% enzyme activity in comparison to WT LTC₄S, showed that the neutral polar side chain of Gln-31 partly compensated for the role of the positively charged guanidino side chain of Arg-31 (Fig. 2A). Gln-31 cannot interact directly with an epoxide group such as Arg-31 due to the side chain being shorter than that of the arginine residue; thus, Gln-31 might affect the epoxide group through the hydrated water molecule(s) of the polar side chain of Gln-31. In addition, the drastically decreased enzymatic activity of the R31A and R31L mutants shows that the bulk water molecules are unable to compensate for the role of Arg-31 on the epoxide group. Furthermore, the R31E mutant with decreased enzyme activity shows that the negatively charged side chain of Glu-31 cannot compensate for the role of Arg-31 with the positively charged side chain, in contrast to Gln-31. The negatively charged side chain of Glu-31 makes the hydrated water molecules electrostatically negative. Therefore, the electrostatically negative water molecule hardly interacts with the negatively polarized epoxide oxygen or the negatively charged alkoxide group formed in the course of the catalysis. A polar functional group with fewer degrees of freedom of motion around the epoxide group of LTA₄-Me, such as the guanidino side chain of Arg-31 or the hydrated water molecule of Gln-31, makes an effective hydrogen bond with the epoxide group so as to accelerate the nucleophilic attack. Arg-31 probably fits as a catalytic amino acid residue for LTA₄-Me.

In comparison with WT LTC₄S, the R31A mutant sustained a comparable $K_m$ for LTA₄-Me despite a much lower $k_{cat}$ (Table 2 and Fig. 3D). This suggests that Arg-31 does not contribute to the LTA₄-Me binding, in contrast to the binding activity of Arg-104 for GSH.

LTC₄S has certain unique features as a nuclear membrane-embedded enzyme responsible for LTC₄ biosynthesis. In our proposed catalytic mechanism, Arg-31 and Arg-104 function as the catalytic amino acid residues by forming a hydrogen bond with LTA₄ and GSH, respectively. Arg-104 forming hydrogen bonds with the thiol group of GSH facilitates the formation of the thiolate anion as the nucleophile, and the thiolate anion attacks the epoxide carbon of LTA₄. Furthermore, Arg-104 also substantially contributes to GSH binding. Arg-31, which increases the electrophilicity of the epoxide carbon of LTA₄ through a hydrogen bond with the epoxide oxygen, makes the nucleophilic attack easy at the initial step. Concomitantly with the development of the nucleophilic attack, the protonated guanidino side chain of Arg-31 neutralizes the negative charge growing on the epoxide oxygen through the hydrogen bond. At the final step of the nucleophilic attack, Arg-31 may donate a proton at the generated alkoxide from the epoxide oxygen to transform the alkoxide group to the hydroxyl group of the product, LTC₄, because alkoxide, the pKₐ of which is 16–18, is more basic than the guanidino side chain of the arginine residue (54).

The results of the enzyme assay on the mutants of Arg-31 are consistent with the current LTA₄ binding model but not sufficient to conclude that the model is correct. The model is speculated based on the crystal structure of the LTC₄S complex with the hydrocarbon tail of dodecyl maltose at the putative LTA₄ binding site. Determination of the complexed structure of LTC₄S with LTA₄ is required to understand the LTA₄ binding mode and functional role of Arg-31; however, the crystal structure analysis of LTC₄S complexed with LTA₄ is a great challenge because LTA₄ is not stable.

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