Leukotriene B₄ 12-hydroxydehydrogenase catalyzes the conversion of leukotriene B₄ into its biologically less active metabolite, 12-oxo-leukotriene B₄. This is an initial and key step of metabolic inactivation of leukotriene B₄ in various tissues other than leukocytes. Here we report the cDNA cloning for porcine and human enzymes from kidney cDNA libraries. A full-length cDNA of the porcine enzyme contains an open reading frame consisting of 987 base pairs, corresponding to 329 amino acids. The human enzyme showed a 97.1% homology with the porcine enzyme. Northern blotting of human tissues revealed its high expression in the kidney, liver, and intestine but not in leukocytes. The porcine enzyme was expressed as a glutathione S-transferase fusion protein in Escherichia coli, which exhibited similar characteristics with the native enzyme. Because the enzymes have a homology, in part, with NAD(P)⁺-dependent alcohol dehydrogenases, a site-directed mutagenesis study was carried out. We found that three glycines at 152, 155, and 166 have crucial roles in the enzyme activity, possibly by producing an NAD(P)⁺ binding pocket.

Leukotriene B₄ (LTB₄) is a potent chemotactic and proinflammatory factor produced in various tissues (1–4). Arachidonic acid, released from the cell membrane by various stimuli, is converted to 5-hydroperoxyicosatetraenoic acid and LTA₄ by 5-lipoxygenase (5–8). LTB₄ is biosynthesized from LTA₄ by the action of LTA₄ hydrolase (9–13). In human polymorphonuclear leukocytes, LTB₄ is converted to 20-hydroxy-LTB₄ by a cytochrome P-450-dependent enzyme. Because the enzymes have homology, in part, with NAD(P)⁺-dependent alcohol dehydrogenases, a site-directed mutagenesis study was carried out. We found that three glycines at 152, 155, and 166 have crucial roles in the enzyme activity, possibly by producing an NAD(P)⁺ binding pocket.

EXPERIMENTAL PROCEDURES

Materials—LTB₄ was kindly donated by Ono Pharmaceutical Company (Osaka). EDTA-Na₂, dithiothreitol, pepstatin-A, and phenylmethylsulfonyl fluoride were purchased from Wako Pure Chemicals (Osaka). NADP⁺ was obtained from Sigma.

N-terminal and Internal Amino Acid Sequences of LTB₄ 12-Hydroxydehydrogenase—The porcine LTB₄ 12-hydroxydehydrogenase was purified as described previously (22). The purified enzyme (100 µg) was digested with 5 µg of trypsin in 100 µm Tris-HCI, pH 8.5, at 37 °C for 8 h. Digested fragments were purified by reversed phase HPLC using a Pharmacia Smart System equipped with a µRPC C₁₈ column (2.1 × 100 mm). The digested enzyme was injected onto a µRPC C₁₈ column previously equilibrated with 0.1% trifluoroacetic acid in water and eluted by a linear gradient to 80% acetonitrile with 0.1% trifluoroacetic acid for 3.8 ml at a flow rate of 100 µl/min. The eluted peptide fragments were monitored at 215 nm, and 31 fractions were collected. LTB₄ 12-hydroxydehydrogenase (5 µg) and six of 31 peptide fragments (Fractions 8, 19, 24, 25, 27, and 37) were loaded on polyvinylidene difluoride membranes with Prospin® (Perkin Elmer) and sequenced by Edman degradation using an automated protein sequencer PPSQ-10 (Shimadzu, Kyoto). SWISS PROT protein data base was used to search for homologous proteins using a BLAST program (23).

cDNA Cloning of Porcine LTB₄ 12-Hydroxydehydrogenase—Degenerate reverse transcriptase-polymerase chain reaction using mixed oligonucleotide primers was performed to obtain a partial cDNA fragment for screening of the library. Mixed oligonucleotide primers were designed according to the amino acid sequences of N-terminal and Fraction 19. Each primer was synthesized by Sawadaya Technology (Tokyo), and the sequences of sense and antisense primers were 5'-GGGGCCACCT-GGCTCAG/GTC/CAT/T(CT)TT/T(C)G-3' (corresponding to the peptide of Fraction 19, 30mers), respectively.

Total RNA was prepared from the porcine kidney by a cesium chloride-urea density gradient method (24). Poly(A)⁺ RNA was purified using Oligotex®-dT30 Super (Roche)apanesing to the manufacturer's manual. An oligo(dT)₁₇–₁₈ (Pharmacia)-primed cDNA was synthesized from 1 µg of poly(A)⁺ RNA by an M-G3 murine leukemia virus reverse transcriptase (Life Technologies Inc.).

The conditions of polymerase chain reaction were as follows: denaturation at 94 °C for 1 min, annealing at 50°C for 2 min, and elongation at 72°C for 3 min. After 5 cycles, the annealing temperature was lowered to 55°C. After 30 cycles of polymerase chain reaction, the products were ethanol-precipitated and separated on a 1% agarose gel, and 4 different bands were recovered from the gel using a QIAGEN gel...
purification kit. Each band was ligated into a T-vector (Promega) by a T
DNA ligase, and the resulting constructs were transformed into
E. coli strain JM 109 (Competent high II, TOYOBO, Tokyo). Plasmids were purified by an alkaline lysis method and sequenced with an ABI automated DNA sequencer 373A (Perkin Elmer). A band of 220 base pairs encoded the 5′ end of the cDNA and was used as a probe to screen the library.

An oligo(dT)-primed λ Zap II (Stratagene) porcine kidney cDNA library was constructed from 4 µg of poly(A)⁺ RNA with Superscript II Choice System (Life Technologies Inc.) according to the manufacturer’s manual. The library yielded 1.6 × 10⁷ independent clones. Full-length cDNA clones were obtained by a plaque hybridization method. The cDNA clones were transferred to 0.8-µm filter paper and the filters, and then the filters were alkali-denatured and fixed by baking at 80 °C for 2 h. The insert cDNA was digested out from the vector, randomly labeled by [³²P] dCTP using a Multiprime Labeling System (Amersham Corp.), and used as a probe for hybridization. After hybridization in Rapidhyb solution (Amersham Corp.) at 65 °C for 8 h, each filter was washed extensively three times in 0.1 × SSC, 0.1% SDS at 65 °C for 20 min. Three rounds of screening gave three positive clones named pBDH 9, 14, and 15. Each clone was excised in vivo into a pBluescript II SK (−) phagemid by ExAssist helper phage (Stratagene), mapped using various restriction enzymes, and sequenced as described previously. All the clones showed the same restriction patterns, and sequencing confirmed that these three clones code for full-length cDNA clones. Terminal nucleotides of full-length cDNA prepared by exonuclease III from pBDH 15, and both strands were sequenced. In addition, six internal sequencing primers were synthesized, and the sequences were confirmed.

**cDNA Cloning of Human LTβ 12-Hydroxydehydrogenase**—Human cDNAs of LTβ 12-hydroxydehydrogenase were isolated from a human kidney cDNA library (Clontech) by a cross-hybridization method with a porcine full-length cDNA (pBDH 15) as a probe. 6 × 10⁵ clones were transferred to Biodyne nylon membranes (Pall) and hybridized at 55 °C for 12 h with a [³²P] dCTP-labeled full-length porcine cDNA (pBDH 15). Each filter was washed three times in 2 × SSC, 0.1% SDS at 55 °C for 10 min. Three rounds of screening gave two phage clones (A149E and A149V), and 8, which were then purified, digested by EcoRI, subcloned into a pBluescript II SK (+) vector, and sequenced. Homology search was performed against the GenBank, EMBL, and SCOP (structural classification of proteins) data bases using a BLAST program (23). The three-dimensional data of crystallized proteins were obtained from the SCOP data base and analyzed using a RMS program (25). An open reading frame and the deduced amino acid sequence were determined by a Genetyx Mac® 6.0.2 software (Software Development, Tokyo, Japan).

Northern blot analysis—Human multiple tissue Northern blots (2 µg of poly(A)⁺ RNA per lane, Clontech) were hybridized with a [³²P] dCTP-labeled full-length human LTβ 12-hydroxydehydrogenase cDNA (hBDH 4) or a human β-actin cDNA (3 h in Rapidhyb solution). Hybridized membranes were washed for 15 min once in 3 × SSC, 0.1% SDS and for 20 min twice in 0.1 × SSC, 0.1% SDS at 65 °C. Autoradiography was subjected to a Bas-2000 system analyzer (Fuji Film, Tokyo, Japan).

Expression of LTβ 12-Hydroxydehydrogenase as a GST Fusion Protein—The porcine cDNA insert was digested out from pBDH 15 by EcoRI and subcloned into a Pharmacia pGEX-1 expression vector (pGEX-TLB12DH). An E. coli strain JM 109(th) TOYOBO) was transformed by heat shock, and then the recombinant protein was induced with 0.1 m isopropyl-1-thio-β-D-galactoside. The procedure was basically as described in the manufacturer’s manual, except that the protein was induced at 20 °C overnight with 0.1 m isopropyl-1-thio-β-D-galactoside (Promega). Resuspended PBS (–) contained 0.1% EDTA-Na₂, 1.0% dithiothreitol, 0.1% phenylmethylsulfonyl fluoride, 0.2 µg/ml pepstatin-A, 2 µg/ml leupeptin, and disrupted by sonication. The sonicates were centrifuged for 10 min at 10,000 × g and 200 µl (for 1 liter of E. coli culture) of GSH-sepharose (Pharmacia) was added to the supernatant. After washing with PBS (−), the protein was eluted in 50 mM Tris-HCl, 1% g/ml pepstatin-A, 0.1% mercaptoethanol and the purity was checked by SDS-PAGE. The 7.5% polyacrylamide gel was stained with Coomasie Brilliant Blue G, and the quantity of the recombinant protein was measured by scanning with bovine serum albumin as a standard. The enzyme activity of the recombinant protein was measured as described previously (22).

**Antibody Production**—A rabbit against LTβ 12-hydroxydehydrogenase—A peptide (ESELETLKAKSEP, corresponding to the amino acid residues 197–210 of the porcine enzyme) was synthesized as a multiple antigen peptide (Fmoc MAP-peptide, B-Branch, Applied Biosystems). An aliquot of 0.5 mg of the peptide was emulsified with an equal volume of Freund’s complete adjuvant and injected into 3–9-month-old female New Zealand white rabbits. After three immunizations at one-month intervals, blood samples were collected and the serum was obtained by centrifugation. The anti-enzyme serum was purified by affinity chromatography. The recombinant LTβ 12-hydroxydehydrogenase (1 mg) was coupled to Sepharose (Pharmacia) (50 mW sodium bicarbonate buffer, pH 9.0) at 25 °C for 10 h. The Sepharose was loaded on a Poly-Prep Chromatography Column (Bio-Rad). After washing with 5 ml of the coupling buffer, 5 ml of the blocking buffer (50 mM Tris-HCl, 1% NaCl, 1% Trition X-100, pH 7.5), the column was equilibrated with PBS (−). 2 ml of anti-serum was applied on the column and allowed to stand at 25 °C for 1 h. After washing the column with 10 ml of PBS (−), 30 ml of the wash buffer, 30 ml of PBS (−), 30 ml of 0.15 M NaCl, the antibody was eluted in 2 ml of the elution buffer. The eluate was immediately neutralized with 100 µl of 1× Tris-HCl, pH 8.0. The concentration of the purified antibody was 236 mg/ml. The affinity-purified antibody is termed λ2 antibody hereafter.

Site-directed Mutagenesis of the Putative NAD-binding Domain—A mutagenesis study was performed by an oligonucleotide-deleted mutagenesis method (26) using a Transformer® site-directed mutagenesis kit (Clontech). The mutagenetic primers were designed as follows: M1 (A149V), 5′-GATGTTAATGCTTGACGACGAGGG-3′; M2 (A150V), 5′-GTTAATGCGGTAGCAGGGGCC-3′; M3 (G151V), 5′-GGCGGCAGCTGGGGTTGTCCT-3′; M4 (G155V), 5′-GGGGCCGGTTGTTCTCTGTCGTGTCCTGTGGTCGACAGCATCCTGAG-3′; M5 (G159V), 5′-CTCTGTCGTGGTCCAGATCGC-3′; M6 (G169V), 5′-CGAGCTCAAGTGCTGAAAGTGTT-3′; M7 (A149V, A150V, G151V, G155V, G159V), 5′-GATGTTAATGCTTGACGACGAGGGCCGTTAGCTGTCACATCGTTGGAATCCGACAGCATCCTGAG-3′; and M8 (A149E), 5′-GATGTTAATGCGGTAGGAGCAGGG-3′. Each mutagenetic primer (10 ng) and a selection primer (10 ng, Aat II/ EcoRV, 5′-GTGACCACTTGATCTGAAACATC-3′) were annealed simultaneously to 10 ng of pdX-TLB12DH, and the first strand was synthesized with 4 units of T7 DNA polymerase and 6 units of T7 DNA ligase in 30 µl at 37 °C for 2 h. Aat II (20 units) was added to selectively linearize the parental DNA. 40 µl of the electocompetent BMH71-18 mut strain (Clontech, CA) was transformed with 2 µl of 5-fold diluted reaction mixture using a Gene Pulser Unit (Bio-Rad). The condition of electroporation was 1.8 kV, 25 microfarad, 100 Ω. After shaking the culture in 10 ml of TB medium overnight, the plasmids were recovered by an alkaline lysis method, and 100 ng of plasmids were digested with Aat II (10 units) again. J. M 109 cells were transformed with 10 ng of digested plasmids by heat shock, and colonies were isolated. Each mutated plasmid was sequenced entirely to check for unexpected mutations. The mutant proteins were purified as GST fusion proteins as described previously. Purified proteins (1 µg/lane) were separated on a 7.5% SDS-PAGE gel and visualized as described (20). The antibody hereafter. The Vₘₜₐₓ and Kₗₜₐₜ values against LTβ 4 and NAD⁺ were determined as described previously (22) six times in three independent experiments.

**RESULTS**

cDNA Cloning of Porcine and Human LTβ 12-Hydroxydehydrogenase—Screening of 1.0 × 10⁶ porcine clones with the probe coding for the 5′ end gave three independent positive clones, pBDH 9, 14, and 15. Three clones were excised in vivo into pBluescript II SK (−) and mapped using several restriction enzymes. The inserts gave an identical restriction map, and DNA sequencing confirmed that these three clones coded for full-length cDNAs of LTβ 12-hydroxydehydrogenase. pBDH 15 was further sequenced by deletion with exonuclease III and 6 internal sequencing primers. Screening of a human kidney cDNA library (6 × 10⁶ clones) with pBDH 15 gave two independent clones, hBDH 4 and 8, which were identical. The primary structures of porcine and human LTβ 12-hydroxydehydrogenases are shown in Figs. 1 and 2. The deduced amino acid sequences of the porcine enzyme contain all the amino acid sequences of seven peptide fragments obtained from the native porcine kidney enzyme (Fig. 1). The cDNA of pBDH 15
contained a polyadenylation signal after the stop codon (Fig. 1), showing that it codes for a full-length LTB₄ 12-hydroxydehydrogenase. pBDH 15 contains an open reading frame of 987 base pairs and codes for 329 amino acids. The calculated Mr of the porcine enzyme is 35,761, a value similar to that of the native enzyme (22). Because hBDH₄ and 8 lack the stop codon, the human enzyme seems to have additional amino acids in the C-terminal. Several trials to acquire the full-length clones using rapid amplification of cDNA ends were unsuccessful. The identity between the porcine and human enzymes was 83.5% at the amino acid level and 84.7% at the nucleotide level. Amino acid homology was 97.1%. Both porcine and human enzymes showed a high homology (94.5 and 96.1%, respectively) with a previously reported rabbit protein, AdRab-F, which is expressed only in adult rabbit small intestine but not in the baby (27). Function of the AdRab-F protein has not been documented (27), but it seems to be a rabbit homologue of LTB₄ 12-hydroxydehydrogenase judging from the high homology. These three proteins contain a proline-rich motif (250–257 residues) in the C-terminal half (Figs. 1 and 2).

In addition, LTB₄ 12-hydroxydehydrogenases have a weak homology with NAD⁺/NADP⁺-dependent short chain alcohol dehydrogenases (28, 29) and a β-crystallin (30), identity being 30–35%. Especially, a fragment from 149 to 166 of the porcine LTB₄ 12-hydroxydehydrogenase has a relatively high homology (>50%) with these dehydrogenases. Because this domain is considered to be a NADP⁺-binding domain in these dehydrogenases (28, 29), a mutagenesis study was carried out to determine the putative NADP⁺-binding domain of LTB₄ 12-hydroxydehydrogenase (see below).

Northern Blot Analysis—Fig. 3 shows the tissue distribution of mRNA of LTB₄ 12-hydroxydehydrogenase in human tissues. The mRNA is expressed most abundantly in the kidney and liver, followed by small intestine and colon. It was absent in...
human leukocytes. The distribution of mRNA matches the tissue distribution of the enzyme activities studied in various porcine tissues (22).

Expression of LTB₄ 12-Hydroxydehydrogenase as a GST Fusion Protein—The recombinant porcine LTB₄ 12-hydroxydehydrogenase was overexpressed as a GST fusion protein in the E. coli system. When the transformed E. coli was cultured at 37 °C, most of the recombinant protein was precipitated by centrifugation at 10,000 × g. By decreasing the culture temperature to 20 °C, good yields of a soluble protein were obtained. The recombinant protein was purified by affinity column chromatography using a GSH-sepharose column. A typical yield was 3% of the protein from 1 liter of bacterial culture, and the purity of the recombinant protein was 70% judging from SDS-PAGE. The purified GST recombinant protein was detectable as a 62-kDa band by Coomassie Brilliant Blue G-staining, and for anti-GST antibody (data not shown). Among them, M6 and M7 were unstable, showing smaller bands (Fig. 4). All the mutant proteins were detected as 62-kDa bands by Coomassie Brilliant Blue G-staining, and for anti-GST antibody (data not shown). Among them, M6 and M7 were unstable, showing smaller bands (Fig. 4). All the mutants had decreased Vₘₐₓ values, and the remaining activities varied among the mutants (Fig. 5). M2 (A150V) had a value of 91% (wild type) enzyme activity, M1 (A149V) showed 56% activity, and M5 (G159V) 40% activity. M3 (G155V, A150V, G152V, G155V, and G159V, 2%) lost most of the enzyme activity. M8 (A149E) showed 9% activity against the porcine LTB₄ 12-hydroxydehydrogenase (Fig. 4), and anti-GST antibody (data not shown). Among them, M6 and M7 were unstable, showing smaller bands (Fig. 4). All the mutants had decreased Vₘₐₓ values, and the remaining activities varied among the mutants (Fig. 5). M2 (A150V) had an almost full (91% of wild type) enzyme activity, M1 (A149V) showed 56% activity, and M5 (G159V) 40% activity. M3 (G152V, 0%), M4 (G155V, 1%), M6 (G166V, 1%), and M7 (G159V) 40% activity. M3 (G155V, 1%), M4 (G155V, 1%), M6 (G166V, 1%), and M7 (G159V, 1%) lost most of the enzyme activity. M8 (A149E) showed 9% activity against the wild type. There were no significant differences between the wild type and the mutant enzymes in terms of Vₘₐₓ values against LTB₄ and NADP⁺.

All the mutant proteins were detected as 62-kDa bands by Coomassie Brilliant Blue G-staining, and for anti-GST antibody (data not shown). Among them, M6 and M7 were unstable, showing smaller bands (Fig. 4). All the mutants had decreased Vₘₐₓ values, and the remaining activities varied among the mutants (Fig. 5). M2 (A150V) had an almost full (91% of wild type) enzyme activity, M1 (A149V) showed 56% activity, and M5 (G159V) 40% activity. M3 (G152V, 0%), M4 (G155V, 1%), M6 (G155V, 1%), and M7 (G159V, 1%) lost most of the enzyme activity. M8 (A149E) showed 9% activity against the wild type. There were no significant differences between the wild type and the mutant enzymes in terms of Vₘₐₓ values against LTB₄ and NADP⁺ (data not shown).

**DISCUSSION**

LTB₄ is a potent lipid mediator that activates leukocytes to migrate from vessels, to generate superoxide anions, and to release lysosomal enzymes (3). This potent mediator is produced in various tissues like the kidney (21, 31, 32) or skin (33–35) under pathophysiological conditions.

The metabolism of LTB₄ has been intensively studied in leukocytes. Human polymorphonuclear leukocytes convert LTB₄ into 20-hydroxy-LTB₄ by a microsomal NADPH-dependent cytochrome P-450 LTB₄ 12-hydroxydehydrogenase (14, 36–41). 20-Hydroxy-LTB₄ is further metabolized to 20-carboxy-LTB₄ (42). 20-Hydroxy- and 20-carboxy-LTB₄ are 10–30 times less active than LTB₄ (43, 44).

There is another group of LTB₄ metabolites. Porcine leukocytes converts LTB₄ to 10,11-dihydro-LTB₄, 10,11-dihydro-12-oxo-LTB₄, and 10,11-dihydro-12-epi-LTB₄ (37, 45–47). Wainwright and Powell (48) extensively studied the mechanism and found that LTB₄ is first converted to 12-oxo-LTB₄ by a microsomal NADPH-dependent 12-hydroxydehydrogenase and then to 10,11-epi-LTB₄ by a cytosolic NADH-dependent 10,11-reductase in porcine polymorphonuclear leukocytes (48). We purified LTB₄-specific 12-hydroxydehydrogenase from porcine kidney (22) and found that it was different in nature from the porcine polymorphonuclear leukocyte enzyme (48). The purified kidney enzyme also converts LTB₄ to 12-oxo-LTB₄, but it is a cytosolic enzyme and utilizes NADP⁺ as a cofactor (22).

**Fig. 2.** Amino acid alignment of LTB₄ 12-hydroxydehydrogenases and AdRab-F protein. The human sequence is considered to be partial. The hypothetical AdRab-F protein (27) was also aligned as rabbit. The asterisk indicates amino acids that are identical among three species. - indicates amino acids that are identical in two species.
A similar conversion of LTB₄ was reported in the human lung (49), kidney (50), keratinocytes (51), and the guinea pig kidney and liver. The enzyme purified from the porcine kidney is a monomeric protein with an Mᵣ of 35,761, which agrees well with that of the native enzyme. In addition, we obtained a cDNA of the human enzyme by cross-hybridization with the porcine cDNA. The primary structures of the porcine and human enzymes are similar, with an amino acid homology of 97.1%. Both enzymes were highly homologous (94.5 and 96.1%) with a rabbit AdRab-F hypothetical protein (Fig. 2), the mRNA of which was expressed only in the adult rabbit and not in the baby (27). The function of AdRab-F protein has not been reported, but it seems to be a rabbit homologue of LTB₄ 12-hydroxydehydrogenase. Further studies are required to determine the developmental change of the expression of LTB₄ 12-hydroxydehydrogenase.

The expression of LTB₄ 12-hydroxydehydrogenases in the kidney and liver, followed by colon and small intestine (Fig. 3). It is important to note that the mRNA is not expressed in the human leukocytes where the ω-oxidation pathway is present.

LTB₄ 12-hydroxydehydrogenases were homologous with other NAD⁺/NADP⁺-dependent short chain alcohol dehydrogenases. Although the total homology was 35% or less, there was a relatively highly homologous domain (Fig. 6). Among these homologous proteins, three enzymes were crystallized, and the structures were well studied (53–55). Crystal structure analyses revealed that this domain forms a compact β-sheet-α-helix-β-sheet structure and was determined to form a NAD⁺/NADP⁺-binding domain (Fig. 6). An acidic residue adjacent to this domain is supposed to bind to the 2’ and 3’ hydroxyl groups of the adenine ribose of NAD⁺/NADP⁺ (29). In addition, mutagenesis studies of the other dehydrogenases indicated that the GXGXXG(A/A)XXGXXXGG consensus sequence is important to maintain a close contact between the coenzyme and the enzyme by forming an α-helix structure (29, 56). By changing two Gly in this domain of NAD⁺-dependent pyruvate dehydrogenase to Ala, the enzyme activity was decreased (57). This domain is highly conserved in the porcine and human LTB₄ 12-hydroxydehydrogenases and AdRab-F hypothetical protein, and the consensus sequence is 149AAXGXXGXXXGG-XXGXXG [Figs. 2 and 6].

To determine which amino acids are required for the enzyme activity, a site-directed mutagenesis was carried out. Ala¹⁴⁹, Ala¹⁵⁰, Gly¹⁵², Gly¹⁵⁵, Gly¹⁵⁹, and Gly¹⁶⁶ were changed into Val, which has a longer side chain than Ala and Gly, or to Glu, which is negatively charged, and the enzyme activities were measured. M₆ (G166V) and M₇ (A149V, A150V, A150V, G152V, G155V, G159V, and G159V) mutants readily cleaved into shorter peptides, as shown in Fig. 4. The Kₘ values against LTB₄ and NAD⁺⁺ of the recombinant wild type enzyme were 20 μM and 10 μM, respectively. Because these values of the native enzyme purified from the porcine kidney were 10 μM and 1 μM (22), respectively, the recombinant protein may contain a slight influence of the enzyme instability and degradation, the quantities of mutant proteins were standardized on Coomassie Brilliant Blue G-stained SDS-PAGE gels (Fig. 4). The enzyme activities of mutant proteins were measured as the relative activities toward the wild type enzyme.

Fig. 5 summarizes six experiments from three different purifications. M₃ (G152V), M₄ (G155V), M₆ (G156V), and M₇ (A149V, A150V, G152V, G155V, and G159V) mutants lost most of the enzyme activity. M₂ (A150V) mutant exhibited a full enzyme activity, whereas about half and 90% of the activities were lost in M₁ (A149V) and M₈ (A149E) mutants, respectively. The Kₘ values for NAD⁺⁺ of M₁, M₅, and M₈ mutants were not significantly different from that of the recombinant wild type enzyme, although the Vₘₐₓ values were all decreased.
Similar results were obtained from the mutagenesis study in the short-chain alcohol dehydrogenase (58). These results suggest that the longer side chain of Val may inhibit the NADP+ binding in G152V, G155V, and G166V mutants. Because the enzyme activity remains moderate in these mutants. In contrast, by changing Ala149 to Glu, most of the enzyme activity was lost, possibly due to the negative charge of Glu. These results indicate that Gly152, Gly155, and Gly166 of LTB4 12-hydroxydehydrogenase are essential for the enzyme activity, probably by forming an NADP+ binding pocket (Fig. 6). As seen in Fig. 6, these three Gly are well conserved among LTB4 12-hydroxydehydrogenases and other homologous proteins, suggesting that these Gly are important. Ala149 and Gly159 seem to play some roles in the enzyme activity but are not essential. Ala159 seems to have only a little role in the enzyme activity.

There is a proline-rich motif that is conserved among three species in the C-terminal half of LTB4 12-hydroxydehydrogenase (Fig. 2, 250–257 residues). The proline-rich motif was reported to play crucial roles by binding src homology 3 (SH3) domains in the signal transduction system of tyrosin-kinase receptors (59). Recently, the binding of proline-rich domains to SH3 domain was reported to be involved in the translocation and activation of 5-lipoxygenase (60), which catalyzes the initial step of biosynthesis of leukotrienes. The role of the proline-rich domain of LTB4 12-hydroxydehydrogenase remains to be clarified.

In conclusion, LTB4 12-hydroxydehydrogenase cDNAs were isolated from the porcine and human kidney, and their primary structures were identified. Northern blotting revealed that the mRNA was expressed in the kidney, liver, small intestine, and colon but not in leukocytes. By a site-directed mutagenesis study, we found that three Gly residues at 152, 155, and 166 play important roles in the enzyme activity. The acquisition of the cDNA and the antibody paves the way for the further analysis of the cellular localization and the biological significance of the enzyme under various physiological and pathological conditions.

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Fig. 6. Amino acid alignment of NAD+/NADP+-binding domains of LTB4 12-hydroxydehydrogenase and other homologous proteins. The amino acid sequences of the porcine and human LTB4 12-hydroxydehydrogenases (LTB412DH) are aligned with rabbit Adarabin protein (27) and other homologous proteins. CRZ (MOUSE), mouse c-crystallin (30); ADH (YEAST), Saccharomyces cerevisiae alcohol dehydrogenase 1 (61); FAS (RAT), rat fatty acid synthase (62); PKS (S. hygroscopos), Streptomyces hygroscopos polyketide synthase (63); QOR (E. coli), E. coli quinone oxidoreductase (54) (SCOP entry 1gor); HDC (S. hygroscopos), Streptomyces hygroscopos 3-alpha-20-hydroxysteroid dehydrogenase (53) (SCOP entry 2hsd); and ADH (HORSE), horse alcohol dehydrogenase (55) (SCOP entry 2hs). The bold letters show amino acids identical with the porcine LTB4 12-hydroxydehydrogenase. The underlined letters show amino acids that form β-helix structures, and the letters in the shaded boxes are α-helix structures derived from the crystal structure analyses of three proteins (QOR, HDC, and ADH). Three Gly in the open boxes (152, 155, and 166) play important roles in porcine LTB4 12-hydroxydehydrogenase activity and are well conserved among these proteins shown in this figure.
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