Acylase-Catalyzed Deacetylation of Haloalkene-Derived Mercapturates

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Mercapturates (*S*-substituted *N*-acetyl-L-cysteines) are terminal metabolites formed by the glutathione-dependent metabolism of electrophilic xenobiotics, including haloalkenes. Acylases catalyze the hydrolysis of *N*-acyl-L-amino acids, including many xenobiotic-derived mercapturates, to give fatty acids and amino acids as products. Although several acylases have been identified, the acylases that catalyze the deacetylation of the haloalkene-derived mercapturates have not been identified and characterized. Acylase I catalyzes the deacetylation of some haloalkene-derived mercapturates, including *S*-(1,1,2,2-tetrafluoroethyl)-*N*-acetyl-L-cysteine, *S*-(2-chloro-1,1,2-trifluoroethyl)-*N*-acetyl-L-cysteine, and *S*-(2-bromo-1,1,2-trifluoroethyl)-*N*-acetyl-L-cysteine [Uttamsingh, V., et al. (1998) *Chem. Res. Toxicol.* **11**, 800−809]. In the studies presented here, we identified a rat kidney acylase that catalyzed the hydrolysis of the haloalkene-derived mercapturates *S*-(1,2-dichlorovinyl)-*N*-acetyl-L-cysteine, *S*-(1,2,3,4,4-pentachloro-1,3-butadienyl)-*N*-acetyl-L-cysteine, and *S*-(2,2-dibromo-1,1-difluoroethyl)-*N*-acetyl-L-cysteine. The substrate selectivity and amino acid sequence of the purified rat kidney acylase were studied. Although the sequence of the purified rat kidney acylase was somewhat identical with that of aspartoacylase, it did not catalyze the hydrolysis of *N*-acetyl-L-aspartate.

Introduction

Halogenated alkenes are widely used in various industrial processes, and some are hepatotoxic, nephrotoxic, cytotoxic, and mutagenic. Several haloalkenes undergo metabolism via the cysteine conjugate β-lyase pathway (*1*, *2*). Glutathione transferase-catalyzed conjugation of haloalkenes with glutathione is the first step in the cysteine conjugate β-lyase pathway and takes place mainly in the liver. The haloalkene-derived glutathione *S*-conjugates formed in the liver are excreted in bile and metabolized by γ-glutamyltransferase and dipeptidases to cysteine *S*-conjugates that may be transported to the kidney and undergo cysteine conjugate β-lyase-dependent bioactivation. Alternatively, the cysteine *S*-conjugates may be reabsorbed by the liver and *N*-acetylated by *N*-acetyltransferases. The mercapturates thus formed may be accumulated in the kidney by the renal organic anion transporter and either excreted in the urine or deacetylated by acylases to yield cysteine *S*-conjugates, which may be bioactivated by cysteine conjugate β-lyase to cytotoxic metabolites. Cysteine conjugate β-lyase is a pyridoxal phosphate-dependent enzyme that requires substrates with a free amino group. Hence, haloalkene-derived mercapturates require deacetylation before cysteine conjugate β-lyase-catalyzed formation of reactive intermediates can occur. Thus, the balance between the *N*-acetylation and deacetylation reactions is an important determinant of the amounts of cysteine *S*-conjugates present in the kidney and, hence, an important factor in the observed nephrotoxicity of haloalkenes.

Several studies indicate that the acylases play a role in xenobiotic detoxication and bioactivation. Bray and James (*3*) observed the deacetylation of mercapturates in vivo in rabbits, rats, and guinea pigs and by rabbit, rat, and guinea pig kidney and liver tissue extracts. Rat kidney cytosol catalyzes the deacetylation of, and increases the mutagenicity of, *S*-(pentachlorobutadienyl)-*N*-acetyl-L-cysteine, *S*-(trichlorovinyl)-*N*-acetyl-L-cysteine, and NADCVC;[1] the level of covalent binding of radioactivity from [*butadienyl*-^{14}C]-*S*-(pentachlorobutadienyl)-*N*-acetyl-L-cysteine to rat kidney proteins is also increased by rat kidney cytosol (*4*, *5*). *S*-(1,1,2,2-Tetrafluoroethyl)-*N*-acetyl-L-cysteine, *S*-(2-chloro-1,1,2-trifluoroethyl)-*N*-acetyl-L-cysteine, *S*-(2,2-dichloro-1,1-difluoroethyl)-*N*-acetyl-L-cysteine, and *S*-(2,2-dibromo-1,1,-difluoroethyl)-*N*-acetyl-L-cysteine are deacetylated in rat renal proximal tubular cells (*6*). [*acetyl*-$^{2}H_3$]-*N*-Acetyl-L-cysteine *S*-conjugates are metabolized to unlabeled mercapturates in vivo, indicating significant deacetylation and reacetylation (*7*, *8*).

Several acylases that catalyze the hydrolysis of *N*-acyl-L-amino acids to give fatty acids and amino acids as products have been identified (*9*): acylase I (EC 3.5.1.14, *N*-acylamino acid amidohydrolase), aspartoacylase or acylase II (EC 3.5.1.15, *N*-acyl-L-aspartate amidohydrolase), acyllysine deacylase (EC 3.1.5.17, N^{ϵ}-acyl-L-lysine amidohydrolase), and acylase III. Acylase I shows a preference for neutral, aliphatic *N*-acyl-α-amino acids; aspartoacylase is specific for *N*-acetyl-L-aspartate, and acylase III catalyzes preferentially the *N*-deacetylation of *N*-acyl aromatic amino acids (*10*). Acylase I has been well characterized. Renal acylase I is a zinc-containing enzyme (*11*). cDNAs encoding the complete amino acid sequence of acylase I have been reported (*12*−*14*). Aspartoacylase from bovine brain has been purified and partially characterized (*15*). A deficiency of aspartoacy-

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[1] Abbreviation: NADCVC, *S*-(1,2-dichlorovinyl)-*N*-Acetyl-L-cysteine.

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lase is associated with Canavan disease, an autosomal recessive leukodystrophy characterized by spongy degeneration of white matter; hence, aspartoacylase is thought to play a role in brain biology (*16*, *17*). cDNAs encoding the amino acid sequence of bovine and human aspartoacylase have been reported (*18*). Acylase III has been partially purified from rat kidney (*19*), and a rat liver acylase with substrate selectivities similar to those of rat kidney acylase III has been identified (*20*).

The enzymes responsible for the deacetylation of xenobiotic-derived mercapturates have not been fully identified. Recent studies show that acylase I catalyzes the deacetylation of *S*-alkyl-*N*-acetyl-L-cysteines, including *N*-acetyl-L-methionine and *S*-ethyl-*N*-acetyl-L-cysteine, and some haloalkene-derived mercapturates, including *S*-(1,1,2,2-tetrafluoroethyl)-*N*-acetyl-L-cysteine, *S*-(2-chloro-1,1,2-trifluoroethyl)-*N*-acetyl-L-cysteine, and *S*-(2-bromo-1,1,2-trifluoroethyl)-*N*-acetyl-L-cysteine (*21*).

We report herein the results of studies in which a rat kidney acylase that catalyzed the hydrolysis of the haloalkene-derived mercapturates (NADCVC), *S*-(1,2,3,4,4-pentachloro-1,3-butadienyl)-*N*-acetyl-L-cysteine, and *S*-(2,2-dibromo-1,1-difluoroethyl)-*N*-acetyl-L-cysteine was identified. The acylase was purified from rat kidney cytosol by column chromatography and partially characterized. The data indicate that the substrate selectivity of the purified rat kidney acylase is similar to that of the previously identified rat kidney and liver acylase III.

Materials and Methods

Materials. *N*-Acetyl-L-cysteine, *N*-acetyl-L-methionine, L-methionine, *N*-acetyl-L-tyrosine, and fluorescamine were purchased from Sigma Chemical Co. (St. Louis, MO). *S*-Benzyl-*N*-acetyl-L-cysteine was purchased from Schweizerhall, Inc. (Plainfield, NJ). *N*-Acetyl-L-aspartic acid, trichloroethylene, and hexachloro-1,3-butadiene were purchased from Aldrich Chemical Co. (Milwaukee, WI), and 1,1-dibromodifluoroethylene was purchased from PCR Inc. (Gainesville, FL).

Syntheses. *S*-(1,2-Dichlorovinyl)-*N*-acetyl-L-cysteine was synthesized as described by McKinney et al. (*22*). *S*-(2,2-Dibromo-1,1-difluoroethyl)-*N*-acetyl-L-cysteine was synthesized by a procedure similar to that described for the synthesis of *S*-(2,2-dichloro-1,1-difluoroethyl)-*N*-acetyl-L-cysteine (*23*). *S*-(1,1,2,3,4-Pentachloro-1,3-butadienyl)-*N*-acetyl-L-cysteine was prepared as described by Nash et al. (*24*). The physical constants of the synthesized products were identical with literature values.

Enzyme Purification. Kidneys from male, Sprague-Dawley rats (Pel-freeze, Rogers, AR) were homogenized in 50 mM potassium phosphate buffer (pH 7.4) at 4 °C. All purification steps were performed at 4 °C. The homogenate was centrifuged at 9000*g*, and the supernatant was centrifuged at 100000*g* to obtain the cytosolic fraction. Solid ammonium sulfate was added to the cytosol to 50% saturation, and the mixture was stirred for 1 h and then centrifuged at 9000*g* for 25 min. The precipitated proteins were dissolved in a minimum volume of 10 mM potassium phosphate buffer (pH 7.4) and dialyzed against the same buffer for 24 h. The dialyzed protein solution was concentrated to 32 mL in an Amicon ultrafiltration cell and applied to a DEAE-cellulose column (5.0 cm × 32 cm, Express Ion-D, Whatman, Hillsboro, OR) equilibrated with 10 mM potassium phosphate buffer (pH 7.4). The column was eluted with 700 mL of 10 mM potassium phosphate buffer (pH 7.4) and then with a linear gradient of 10 to 200 mM (∼650 mL) potassium phosphate buffer (pH 7.4). Eluate fractions (8.2 mL) were collected and assayed for protein concentrations and enzyme activities. Fractions with activity with *N*-acetyl-L-cysteine and *N*-acetyl-L-methionine as substrates were separated from the fractions with activity with NADCVC as the substrate. The fractions that had activity with *N*-acetyl-L-cysteine and *N*-acetyl-L-methionine as substrates were purified as described previously (*22*). Fractions with activity with NADCVC as the substrate were combined and concentrated in an Amicon ultrafiltration cell, and 30 mL of the concentrated fraction was applied to a Sephacryl S-200 HR (5.0 cm × 40 cm, Pharmacia, Piscataway, NJ) column equilibrated with 10 mM potassium phosphate buffer (pH 7.4); the column was eluted with the same buffer. Eluate fractions (4 mL) were collected and assayed for protein concentrations and enzyme activities. Fractions with the highest activities were combined, concentrated in an Amicon ultrafiltration cell, and applied to a hydroxyapatite column (1.5 cm × 12 cm, Macro-Prep Ceramic Hydroxyapatite TYPE I, 40 μm, Bio-Rad Laboratories, Hercules, CA) equilibrated with 10 mM potassium phosphate buffer (pH 7.4). Proteins were eluted with the column equilibration buffer, and eluate fractions (2 mL) were collected and assayed for protein concentrations and enzyme activities. Fractions with the highest activity were combined and applied to a Mono-Q HR5/5 column (0.5 cm × 5 cm, Pharmacia), which was eluted with a linear gradient of 10 to 200 mM (∼40 mL) potassium phosphate buffer (pH 7.4) with a FPLC system (Pharmacia, Piscataway, NJ). Eluate fractions (1 mL) were collected and assayed for protein concentrations and enzyme activities. Fractions with the highest activities were analyzed by SDS−PAGE (10% acrylamide). Proteins were visualized by Coomassie Blue staining. The purified rat kidney acylase was stored at 4 °C for 3 months without a significant loss of acylase activity.

Protein concentrations were determined by the method of Bradford (*25*) with bovine serum albumin as the standard.

Acylase Activity Assays. Acylase activity with NADCVC as the substrate was determined by measuring the amount of deacetylated product formed by reaction with fluorescamine (*26*). The reaction mixture (1 mL) contained 0.1−0.5 mL of enzyme solution and 2 μmol of substrate in 50 mM potassium phosphate buffer (pH 7.4). The reaction mixture was incubated at 37 °C for 1 h, and the reaction was stopped by addition of 0.2 mL of 20% trichloroacetic acid. The mixture was allowed to stand for 10 min in an ice bath and was then centrifuged at 500*g* for 10 min. A sample (40 μL) of the supernatant was added to 3.6 mL of 50 mM potassium phosphate buffer (pH 7.4), and the volume was increased to 4 mL by the addition of water. Fluorescamine (300 μL of a solution containing 10 mg of fluorescamine dissolved in 33 mL of acetone) was added to the sample, and the fluorescence intensity (390 nm excitation, 475 nm emission) was measured with a Perkin-Elmer LS-5 fluorescence spectrophotometer (Norwalk, CT). A standard curve was prepared with L-methionine as the analyte.

The deacetylation of *S*-(1,1,2,2-tetrafluoroethyl)-*N*-acetyl-L-cysteine, *S*-(2-chloro-1,1,2-trifluoroethyl)-*N*-acetyl-L-cysteine, *S*-(2-bromo-1,1,2-trifluoroethyl)-*N*-acetyl-L-cysteine, *S*-(2,2-dibromo-1,1-difluoroethyl)-*N*-acetyl-L-cysteine, *S*-(1,1,2,3,4-pentachloro-1,3-butadienyl)-*N*-acetyl-L-cysteine, *S*-benzyl-*N*-acetyl-L-cysteine, *N*-acetyl-L-tyrosine, and *N*-acetyl-L-aspartic acid with the purified rat kidney acylase was assessed by the method described above.

Kinetic Analyses. The kinetics of the deacetylation of NADCVC, *S*-(2,2-dibromo-1,1-difluoroethyl)-*N*-acetyl-L-cysteine, *S*-(1,1,2,3,4-pentachloro-1,3-butadienyl)-*N*-acetyl-L-cysteine, and *S*-benzyl-*N*-acetyl-L-cysteine was studied with the purified rat kidney acylase. For the determination of the K_m and V_{max}, the reaction mixtures contained 5 μg of the purified rat kidney acylase and 1.0−16.0 mM substrate in a final volume of 1 mL of 50 mM potassium phosphate buffer (pH 7.4). The reaction mixtures were incubated at 37 °C for 30 min, and the amounts of deacetylated products that were formed were measured as described above. Experiments were performed in triplicate. The K_m and V_{max} were computed by fitting the data to the Michaelis−Menten equation with the EZ-FIT program (Perrella Scientific, Inc., Conyers, GA).

Determination of the Molecular Mass of the Purified Rat Kidney Acylase. The molecular mass of the purified rat

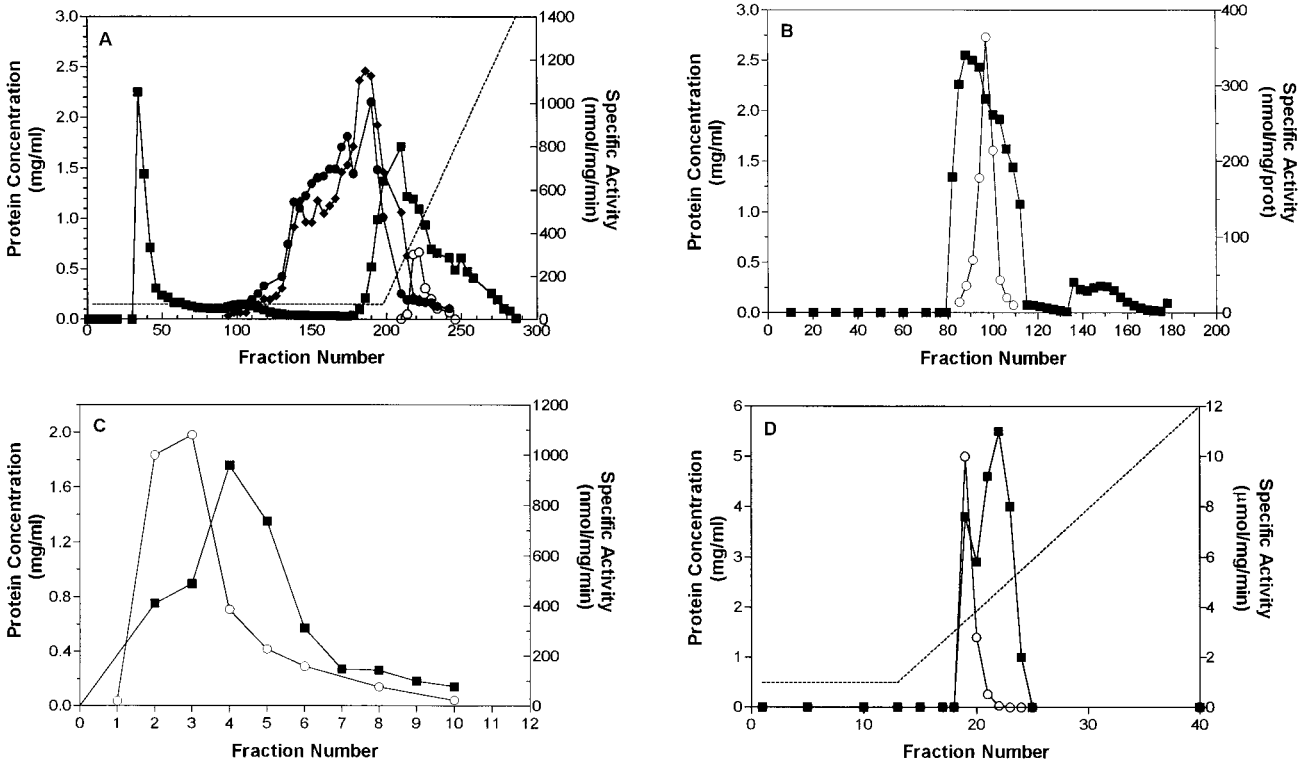

Figure 1. Purification of rat kidney acylase by column chromatography. (A) DEAE-cellulose column chromatography of the 0−50% ammonium sulfate fraction from rat kidney cytosol. The column was eluted with 10 mM potassium phosphate buffer (pH 7.4) and then with a linear gradient (dashed line) of 10 to 200 mM potassium phosphate buffer (pH 7.4). Eluate fractions (8.2 mL) were collected and assayed for protein concentrations (■), and enzyme activities with *N*-acetyl-L-cysteine (●), *N*-acetyl-L-methionine (◆), and NADCVC (○) as substrates were measured as described in Materials and Methods. Fractions 134−206 exhibited high activity with *N*-acetyl-L-cysteine and *N*-acetyl-L-methionine as substrates and were purified as described in Materials and Methods. Fractions 210−235 exhibited activity with NADCVC as the substrate. (B) Sephacryl S-200 HR column chromatography of fractions 210−235 obtained from DEAE-cellulose column chromatography (panel A). The column was eluted with 10 mM potassium phosphate buffer (pH 7.4). Eluate fractions (4 mL) were collected and assayed for protein concentrations (■), and enzyme activity with NADCVC (○) as the substrate was measured as described in Materials and Methods. Fractions 93−100 exhibited high acylase activity. (C) Hydroxyapatite column chromatography of fractions 93−100 obtained from Sephacryl S-200 column chromatography (panel B). The column was eluted with 10 mM potassium phosphate buffer (pH 7.4). Eluate fractions (2 mL) were collected and assayed for protein concentrations (■), and enzyme activity with NADCVC (○) as the substrate was measured as described in Materials and Methods. Fractions 2 and 3 exhibited high acylase activity. (D) Mono-Q column chromatography of fractions 2 and 3 obtained from hydroxyapatite column chromatography (panel C). The column was eluted with 10 mM potassium phosphate buffer (pH 7.4) (fractions 1−12) and then with a linear gradient (dashed line) of 10 to 200 mM potassium phosphate buffer (fractions 13−40). Eluate fractions (1 mL) were collected and assayed for protein concentrations (■), and enzyme activity with NADCVC (○) as the substrate was measured as described in Materials and Methods. Fraction 19 exhibited high acylase activity.

kidney acylase was determined with a HPLC gel filtration column (Bio-Sil SEC-250, 300 mm × 7.8 mm, Bio-Rad Laboratories). The column was equilibrated with 10 mM potassium phosphate buffer (pH 7.4), and the protein was eluted with the same buffer at a rate of 1 mL/min. The molecular mass of the purified rat kidney acylase was estimated by comparison with protein standards (Bio-Rad Laboratories).

Amino Acid Sequencing. Initial attempts to determine the N-terminal sequence were unsuccessful because the protein was N-blocked. The purified rat kidney acylase was subsequently analyzed by SDS−PAGE (10% gel), electrophoretically transferred to a PVDF membrane, and stained briefly with Coomassie blue. The stained protein band on the PVDF membrane was excised for the determination of internal peptide sequences (Proseq Microsequencing Co., Salem, MA). Peptides were generated by digestion of the protein with cyanogen bromide and were sequenced directly or after selection of proline residues with *o*-phthalaldehyde.

Calculations. The molar volumes (*27*) and log *P* (*28*) of the substrates used were calculated with ChemDraw Pro (version 4.5, CambridgeSoft Corp., Cambridge, MA).

Results

Enzyme Purification. Preliminary studies showed that rat kidney cytosol, but not commercially available acylase I, catalyzed the deacetylation of NADCVC, indicating the presence of a distinct acylase in rat kidney cytosol. Rat kidney cytosol was fractionated by ammonium sulfate precipitation; the 0−50% ammonium sulfate fraction obtained exhibited activity with *N*-acetyl-L-cysteine, *N*-acetyl-L-methionine, and NADCVC as substrates (*21*). The acylase-containing fraction was applied to a DEAE-cellulose column. Two distinct sets of acylase-containing fractions were obtained. Fractions 134−206 were eluted with 10 mM potassium phosphate buffer (pH 7.4) and exhibited activity with *N*-acetyl-L-cysteine and *N*-acetyl-L-methionine as the substrates, whereas fractions 210−235 were eluted with approximately 50−60 mM potassium phosphate buffer (pH 7.4) and exhibited activity with NADCVC as the substrate (Figure 1A). The protein fractions that catalyzed the deacetylation of *N*-acetyl-L-cysteine and *N*-acetyl-L-methionine did not catalyze the deacetylation of NADCVC and vice versa. Fractions with activity with NADCVC as the substrate were combined, concentrated, and further purified on a Sephacryl S-200 column (Figure 1B), a hydroxyapatite column (Figure 1C), and finally by Mono-Q anion-exchange FPLC (Figure 1D). SDS−PAGE analysis of

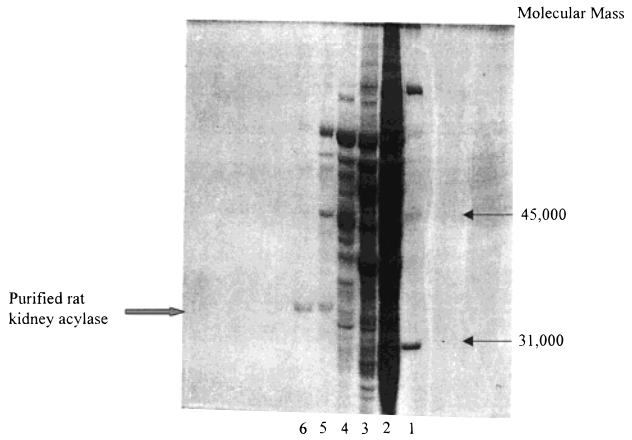

Figure 2. Electrophoretic analysis of purified rat kidney acylase. SDS−PAGE analysis of rat kidney cytosol and fractions obtained from DEAE-cellulose, Sephacryl S-200 HR, hydroxyapatite, and Mono-Q columns (Figure 1) that exhibited activity with NADCVC as the substrate: lane 1, molecular weight markers; lane 2, rat kidney cytosol (20 μg); lane 3, DEAE-cellulose fraction (20 μg); lane 4, Sephacryl S-200 HR column fraction (20 μg); lane 5, hydroxyapatite fraction (10 μg); and lane 6, Mono-Q HR5/5 column fraction 19 (5 μg).

fraction 19 obtained by FPLC (Figure 1D) showed that the enzyme was purified to near homogeneity and had an apparent subunit M_r of 33 000 (Figure 2). Also, Western analysis with polyclonal antipurified rat kidney acylase antibodies showed a single, immunoreactive peptide with an apparent M_r of about 33 000.[2] The enzyme was purified 400-fold (Table 1). The estimated molecular weight of the purified holoenzyme was 55 000 as determined by HPLC gel filtration chromatography (data not shown).

Deacetylation of Haloalkene-Derived Mercapturates. The purified rat kidney acylase catalyzed the deacetylation of *S*-(2,2-dibromo-1,1-difluoroethyl)-*N*-acetyl-L-cysteine, *S*-(1,1,2,3,4-pentachloro-1,3-butadienyl)-*N*-acetyl-L-cysteine, and *S*-benzyl-*N*-acetyl-L-cysteine, but did not catalyze the deacetylation of *S*-(1,1,2,2-tetrafluoroethyl)-*N*-acetyl-L-cysteine, *S*-(2-chloro-1,1,2-trifluoroethyl)-*N*-acetyl-L-cysteine, and *S*-(2-bromo-1,1,2-trifluoroethyl)-*N*-acetyl-L-cysteine. Also, *N*-acetyl-L-tyrosine, but not *N*-acetyl-L-aspartic acid, was deacetylated by the purified rat kidney acylase (data not shown). The V_{max}/K_m value was highest for *S*-(1,1,2,3,4-pentachloro-1,3-butadienyl)-*N*-acetyl-L-cysteine followed by those of NADCVC, *S*-(2,2-dibromo-1,1-difluoroethyl)-*N*-acetyl-L-cysteine, and *S*-benzyl-*N*-acetyl-L-cysteine (Table 2).

Although relatively little data was available, the V_{max}/K_m values for the purified rat kidney acylase-catalyzed deacetylations were not apparently related to calculated molar volume or log *P* values.

Amino Acid Sequence. Initial attempts to obtain the amino acid sequence of the purified rat kidney acylase showed that the N-terminus was blocked. The protein was subsequently degraded with cyanogen bromide, and the sequences of three peptides that were obtained were determined. A sequence of 40 amino acid residues was obtained from one peptide (A), of 20 amino acid residues from a second peptide (B), and of 19 amino acid residues from a third peptide (C). A search of GenBank (Blast search) revealed that the sequence of peptide A was 42% identical with amino acids 220−259 of the human aspartoacylase and with amino acids 220−257 of the bovine aspartoacylase (Figure 3A). The sequence of peptide B was 70% identical with amino acids 87−103 of the human and bovine aspartoacylase (Figure 3B). Residues 3−20 of peptide C were 72% identical with amino acids 47−64 of the potential aspartoacylase protein-coding region from the genome sequence of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803 (Figure 3C).

[2] V. Uttamsingh, R. B. Baggs, D. Krenitsky, and M. W. Anders, manuscript submitted to *Drug Metab. Dispos.*

Discussion

An acylase that catalyzes the deacetylation of the haloalkene-derived mercapturates, NADCVC, *S*-(1,1,2,3,4-pentachloro-1,3-butadienyl)-*N*-acetyl-L-cysteine, and *S*-(2,2-dibromo-1,1-difluoroethyl)-*N*-acetyl-L-cysteine, and *S*-benzyl-*N*-acetyl-L-cysteine, and *N*-acetyl-L-tyrosine was purified from rat kidney cytosol and partially characterized. The purified rat kidney acylase had an apparent subunit M_r of approximately 33 000, and the apparent M_r of the holoenzyme was estimated to be approximately 55 000. The basis for the discrepancy between the observed subunit molecular mass and the molecular mass of the holoenzyme is not apparent. Although the purified rat kidney acylase may exist as a heterodimer, SDS−PAGE analysis showed the presence of a single band with an apparent M_r of 33 000 (data not shown).[2] In addition, Western analysis showed a single, immunoreactive peptide with an apparent M_r of about 33 000. Acylase I is a homodimer with a subunit M_r of 46 000, and aspartoacylase is a monomer with an M_r of 58 000.

The purified rat kidney acylase did not catalyze the deacetylation of the known acylase I substrates *N*-acetyl-L-cysteine and *N*-acetyl-L-methionine and the haloalkene-derived mercapturates *S*-(1,1,2,2-tetrafluoroethyl)-*N*-acetyl-L-cysteine, *S*-(2-chloro-1,1,2-trifluoroethyl)-*N*-acetyl-L-cysteine, and *S*-(2-bromo-1,1,2-trifluoroethyl)-*N*-acetyl-L-cysteine. Also, *N*-acetyl-L-aspartic acid, an aspartoacylase substrate, was not deacetylated by the purified rat kidney acylase. Thus, on the basis of molecular mass and substrate selectivity, the purified rat kidney acylase is distinct from both acylase I and aspartoacylase. Endo (*19*) partially purified an acylase from rat kidney that catalyzes the deacetylation of *N*-acyl aromatic amino acids, including *N*-acetyl-L-tyrosine, and has an M_r of 55 000; the enzyme was termed acylase III. A rat liver acylase that may exist as a homotetramer with a subunit M_r of about 35 000 and whose substrate selectivity is similar to that of the rat kidney acylase III has also been purified and partially characterized (*20*). Thus, the purified rat kidney acylase characterized in this study, which catalyzed the hydrolysis of the acylase III substrates *N*-acetyl-L-tyrosine and *S*-benzyl-*N*-acetyl-L-cysteine, may be identical with the previously described acylase III. Attempts to characterize the purified rat kidney acylase at the molecular level indicate that the internal amino acid sequences of the purified rat kidney acylase are 40−70% identical with the amino acid sequence of bovine and human aspartoacylase; no identity with the amino acid sequence of acylase I was observed. The amino acid sequences of acylase I and aspartoacylase are not related (*15*).

Acylase I, aspartoacylase, and acylase III have distinct substrate selectivities. Previous studies show that the length and degree of branching of the *N*-acyl-L-amino acid side chain have prominent effects on acylase I-catalyzed deacetylation reactions (*21*). A relation between acylase

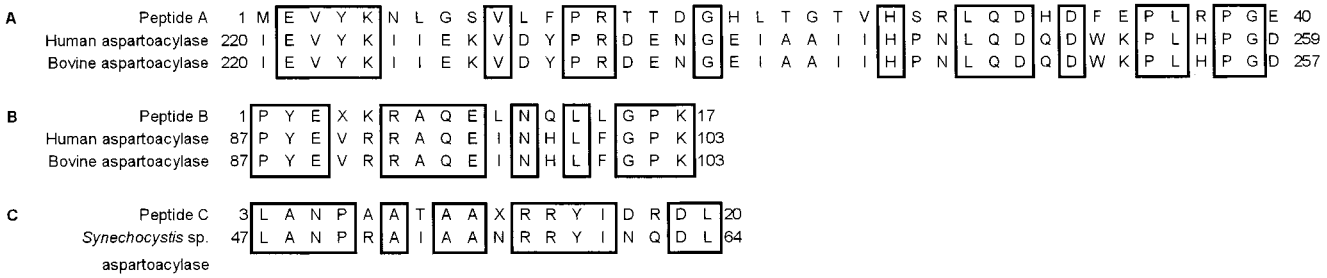

Figure 3. Sequence alignment of internal peptides from the purified rat kidney acylase with human and bovine aspartoacylase amino acid sequences. Rat kidney acylase was purified and analyzed as described in Materials and Methods. Peptides A−C were obtained as described in Materials and Methods. (A) Alignment of the amino acid sequence of peptide A with amino acids 220−259 of the human aspartoacylase and amino acids 220−257 of the bovine aspartoacylase. (B) Alignment of the amino acid sequence of peptide B with amino acids 87−103 of the human and bovine aspartoacylases. (C) Alignment of the amino acid sequence of peptide C with amino acids 47−64 of the potential aspartoacylase protein-coding region from the genome sequence of the unicellular cyanobacterium *Synechocystis* sp. strain PC6803.

Table 1. Purification of Rat Kidney Acylase

				activity with *S*-(1,2-dichlorovinyl)-*N*-acetyl-L-cysteine		
fraction	volume (mL)	protein concentration (mg/mL)	total protein (mg)	specific activity (nmol mg^{-1} min^{-1})	total activity (nmol/min)	purification (*x*-fold)
cytosol	215	45.4	9761	25	244025	
0−50% $(NH_4)_2SO_4$	32	16.5	528	60	31680	2.4
DEAE-cellulose	30	8.5	250	110	27500	4.4
Sephacryl S-200	11	5.0	55.0	254	13970	10.0
hydroxyapatite	12	0.8	9.00	959	8631	38.6
Mono-Q	6	0.08	0.5	10000	5000	400

Table 2. Kinetics of the Purified Rat Kidney Acylase-Catalyzed Deacetylation of Haloalkene-Derived Mercapturates

substrate	V_{max} (μmol mg^{-1} min^{-1})	K_m (mM)	V_{max}/K_m (mL mg^{-1} min^{-1})	molar volume (cm^3/mol)	log *P*
S-(1,2-dichlorovinyl)-*N*-acetyl-L-cysteine	14.3 ± 0.98	8.9 ± 2.5	1.6	55.84	0.90
S-(2,2-dibromo-1,1-difluoroethyl)-*N*-acetyl-L-cysteine	27.0 ± 0.16	15.0 ± 2.4	1.8	63.01	1.44
S-(1,2,3,4,4-pentachlorobutadienyl)-*N*-acetyl-L-cysteine	9.0 ± 0.99	1.1 ± 0.1	8.4	81.27	1.42
S-benzyl-*N*-acetyl-L-cysteine	5.0 ± 0.29	7.5 ± 1.4	0.70	62.19	0.93

I activity and both calculated molar volumes and log *P* was observed. The factors affecting the substrate selectivity of the purified rat kidney acylase are not clear. Although few substrates were studied, the data do not indicate a relationship between calculated molar volumes or log *P* and the rates of purified rat kidney acylase-catalyzed deacetylation reactions. In general, the substrates deacetylated by the purified rat kidney acylase and acylase III have bulkier *N*-acyl-L-amino acid side chains than the substrates deacetylated by acylase I and, hence, higher calculated molar volumes. Acylase III has been described as an *N*-acyl aromatic amino acid deacylase. The observation that the V_{max}/K_m values for the purified rat kidney acylase-catalyzed deacetylation of *S*-(2,2-dibromo-1,1-difluoroethyl)-*N*-acetyl-L-cysteine and NADCVC are similar may indicate that the molar volumes in addition to aromaticity or the presence of π-systems in the *N*-acyl-L-amino acid side chain may determine the substrate selectivity of the purified rat kidney acylase. It should be noted, however, that the 2-(fluoromethoxy)-1,1,3,3,3-pentafluoro-1-propene (Compound A)-derived mercapturates *S*-[2-(fluoromethoxy)-1,1,3,3,3-pentafluoropropyl]-*N*-acetyl-L-cysteine and *S*-[2-(fluoromethoxy)-1,3,3,3-tetrafluoro-1-propenyl]-*N*-acetyl-L-cysteine have bulky *N*-acyl-L-amino acid side chains and are poorly deacetylated both in vivo and in vitro by acylase I and the purified rat kidney acylase (*8*).

The deacetylation of haloalkene-derived mercapturates has been observed both in vitro and in vivo, but the acylases that are involved have not been identified. The acylase-catalyzed deacetylation of mercapturates affords cysteine *S*-conjugates that may undergo cysteine conjugate β-lyase-dependent bioactivation. The data obtained in this study indicate that at least two acylases, acylase I and the purified rat kidney acylase, may influence the intracellular amounts of cysteine *S*-conjugates that are available for cysteine conjugate β-lyase-dependent bioactivation. In addition to the acylase-catalyzed deacetylation, cysteine *S*-conjugate *N*-acetyltransferase-catalyzed acetylation of the cysteine *S*-conjugates would also be expected to influence the concentrations of the cysteine *S*-conjugates. Thus, identification and characterization of acylases and *N*-acetyltransferases involved in the mercapturate pathway can provide useful insights into the cysteine conjugate β-lyase-dependent bioactivation of the cytotoxic, nephrotoxic, and mutagenic cysteine *S*-conjugates.

Further studies aimed at determining the factors that govern the substrate selectivity of the purified rat kidney acylase are warranted. Also, isolation of the cDNA encoding the complete amino acid sequence of the purified rat kidney acylase will help clarify the relationship between aspartoacylase, acylase III, and the purified rat kidney acylase.

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