Specificity of Aminoacylase III-Mediated Deacetylation of Mercapturic Acids

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ABSTRACT:

Trichloroethylene (TCE) and other halogenated alkenes are known environmental contaminants with cytotoxic and nephrotoxic effects, and are potential carcinogens. Their metabolism via the mercapturic metabolic pathway was shown to lead to their detoxification. The final products of this pathway, mercapturic acids or N-acetyl-L-cysteine S-conjugates, are secreted into the lumen in the renal proximal tubule. The proximal tubule may also deacetylate mercapturic acids, and the resulting cysteine S-conjugates are transformed by cysteine S-conjugate β-lyases to nephrotoxic reactive thiols. The specificity and rate of mercapturic acid deacetylation may determine the toxicity of certain mercapturic acids; however, the exact enzymologic processes involved are not known in detail. In the present study we characterized the kinetics of the recently cloned mouse aminoacylase III (AAIII) toward a wide spectrum of halogenated mercapturic acids and N-acetylated amino acids. In general, the Vmax value of AAIII was significantly larger with chlorinated and brominated mercapturic acids, whereas fluorination significantly decreased it. The enzyme deacetylated mercapturic acids derived from the TCE metabolism including N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine (NA-1,2-DCVC) and N-acetyl-S-(2,2-dichlorovinyl)-L-cysteine (NA-2,2-DCVC). Both mercapturic acids induced cytotoxicity in mouse proximal tubule mPCT cells expressing AAIII, which was decreased by an inhibitor of β-lyase, aminooxyacetate. The toxic effect of NA-2,2-DCVC was smaller than that of NA-1,2-DCVC, indicating that factors other than the intracellular activity of AAIII mediate the cytotoxicity of these mercapturic acids. Our results indicate that in proximal tubule cells, AAIII plays an important role in deacetylating several halogenated mercapturic acids, and this process may be involved in their cytotoxicity.

Trichloroethylene (TCE) and other industrial solvents are shown to cause nephrotoxicity and hepatotoxicity, and are probable human carcinogens (Maltoni et al., 1988; Cummings and Lash, 2000). One of the TCE toxicity mechanisms involves the conjugation with glutathione, which is transformed by γ-glutamyltransferase and dipeptidase to cysteine S-conjugates (Dekant et al., 1994; Bernauer et al., 1996; Anders and Dekant, 1998). The formation of cysteine S-conjugates takes place mainly in liver and, to some extent, also in kidney. Cysteine S-conjugates may be acetylated in the liver and kidney into corresponding N-acetyl S-conjugates (mercapturic acids) and transported from the liver into the kidney. In the kidney proximal tubules, mercapturic acids may be secreted or deacetylated by aminoacylases into cysteine S-conjugates (Anders and Dekant, 1994, 1998). The deacetylation reaction may play an important role in the renal nephrotoxicity since cysteine S-conjugates, but not mercapturates, are transformed by cysteine conjugate β-lyases into toxic metabolites (Hayden and Stevens, 1990; Anders et al., 1994; Commandeur et al., 1995). Cysteine S-conjugates also may be transformed by flavoprotein monooxygenases or cytochrome P450 into sulfoxides (Anders and Dekant, 1998; Lash et al., 2003). The flavoprotein monooxygenase-dependent bioactivation of the cysteine conjugate of TCE, S-(1,2-dichlorovinyl)-L-cysteine (1,2-DCVC), was shown to be involved in the toxicity of TCE in rodent and human proximal tubule cells (Lash et al., 2001, 2003).

Several aminoacylases have been described, including aminoacylase I (AAI; EC 3.5.1.14, N-acylamino acid hydrolase), aminoacylase II (AAII or aspartoacylase; EC 3.5.1.15, N-acyl-L-aspartate amidohydrolase), acyllysine deacylase (EC 3.1.5.17, N-acyl- L-lysine amidohydrolase), and aminoacylase III (AAIII, preferentially mediating deacylation of N-acyl aromatic amino acids). AAI has been purified from mammalian kidney and liver (Anders and Dekant, 1994; Giardina et al., 1997) and subsequently cloned (Jacob et al., 1992;
Mitta et al., 1992; Giardina et al., 2000). AAI is a metalloenzyme, which belongs to the Acyl/M20 family. It contains one Zn²⁺ per monomer of molecular mass ~43 kDa. AAI isolated from different sources was shown to be a homodimer (Kordel and Schneider, 1977; Lindner et al., 2003, 2005). AAI was shown to deacetylate several S-alkyl derivatives of N-acetyl-L-cysteine including N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine, N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, and N-acetyl-S-(2-bromo-1,1,2-trifluoroethyl)-L-cysteine (Kordel and Schneider, 1976, 1977; Heese et al., 1988; Anders and Dekant, 1994; Giardina et al., 2000). AAI is a metalloenzyme, which belongs to the AcyI/M20 family. It contains one Zn2⁺, as shown in the above references. AAI was shown to deacetylate several S-alkyl derivatives of N-acetyl-L-cysteine including N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine, N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, and N-acetyl-S-(2-bromo-1,1,2-trifluoroethyl)-L-cysteine (Kordel and Schneider, 1976, 1977; Heese et al., 1988; Anders and Dekant, 1994; Giardina et al., 2000; Mitta et al., 1992; Giardina et al., 2000). AAI is a metalloenzyme, which belongs to the Acyl/M20 family. It contains one Zn²⁺ per monomer of molecular mass ~43 kDa. AAI isolated from different sources was shown to be a homodimer (Kordel and Schneider, 1977; Lindner et al., 2003, 2005). AAI was shown to deacetylate several S-alkyl derivatives of N-acetyl-L-cysteine including N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine, N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, and N-acetyl-S-(2-bromo-1,1,2-trifluoroethyl)-L-cysteine (Kordel and Schneider, 1976, 1977; Heese et al., 1988; Anders and Dekant, 1994; Giardina et al., 2000). AAI is a metalloenzyme, which belongs to the Acyl/M20 family. It contains one Zn²⁺, as shown in the above references. AAI was shown to deacetylate several S-alkyl derivatives of N-acetyl-L-cysteine including N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine, N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, and N-acetyl-S-(2-bromo-1,1,2-trifluoroethyl)-L-cysteine (Kordel and Schneider, 1976, 1977; Heese et al., 1988; Anders and Dekant, 1994; Giardina et al., 2000). AAI is a metalloenzyme, which belongs to the Acyl/M20 family. It contains one Zn²⁺, as shown in the above references. AAI was shown to deacetylate several S-alkyl derivatives of N-acetyl-L-cysteine including N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine, N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, and N-acetyl-S-(2-bromo-1,1,2-trifluoroethyl)-L-cysteine (Kordel and Schneider, 1976, 1977; Heese et al., 1988; Anders and Dekant, 1994; Giardina et al., 2000). AAI is a metalloenzyme, which belongs to the Acyl/M20 family. It contains one Zn²⁺, as shown in the above references. AAI was shown to deacetylate several S-alkyl derivatives of N-acetyl-L-cysteine including N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine, N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, and N-acetyl-S-(2-bromo-1,1,2-trifluoroethyl)-L-cysteine (Kordel and Schneider, 1976, 1977; Heese et al., 1988; Anders and Dekant, 1994; Giardina et al., 2000). AAI is a metalloenzyme, which belongs to the Acyl/M20 family. It contains one Zn²⁺, as shown in the above references. AAI was shown to deacetylate several S-alkyl derivatives of N-acetyl-L-cysteine including N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine, N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, and N-acetyl-S-(2-bromo-1,1,2-trifluoroethyl)-L-cysteine (Kordel and Schneider, 1976, 1977; Heese et al., 1988; Anders and Dekant, 1994; Giardina et al., 2000). AAI is a metalloenzyme, which belongs to the Acyl/M20 family. It contains one Zn²⁺, as shown in the above references. AAI was shown to deacetylate several S-alkyl derivatives of N-acetyl-L-cysteine including N-acetyl-S-(1,1,2,2-

Materials and Methods

Expression and Purification of Mouse AAIII. Mouse AAIII was expressed in human embryonic kidney HEK293T cells as a His₁₀-tagged fusion protein using a pcDNA3.1/His vector (Invitrogen, Carlsbad, CA). The AAIII purification was performed as described before (Pushkin et al., 2004), with minor modifications: ion-exchange chromatography on DEAE-cellulose DE52 (Whatman, Maidstone, Kent, UK) was used instead of Ni-Superflow resin (Novagen, Madison, WI) chromatography. The purified His₁₀-tagged AAIII protein was homogeneous upon polyacrylamide gel electrophoresis (PAGE) in the presence of SDS, and Western blotting with both a mouse kidney AAIII-specific MR-C1 antibody (Pushkin et al., 2004) and a penta-His antibody (Qiagen, Valencia, CA). The fusion protein was untagged using a recombinant enterokinase from Novagen (Pushkin et al., 2004). Before use, the purified untagged AAIII was centrifuged at 200,000 g for 1 h at 4°C in an Optima TLX Ultracentrifuge (Beckman Coulter, Fullerton, CA) to precipitate the aggregated protein.

Because AAIII is an oligomer and its oligomeric structure may affect the enzyme kinetics, we evaluated the oligomeric structure of the purified murine AAIII. PAGE in non-denaturing conditions detected one major and one minor band (Fig. 1A), both reacting with the mouse AAIII-specific MR-C1 antibody used for Western blotting. Size-exclusion chromatography also identified two peaks corresponding to ~75- and ~135-kDa proteins (Fig. 1B). The results of the SDS-PAGE of the cross-linked proteins in these fractions (Fig. 1B) and transmission electron microscopy (Fig. 1C) confirmed the results of size-exclusion chromatography and suggested that the purified murine AAIII is predominantly a tetramer with a small amount of dimers. The tetramers were separated from the dimers using size-exclusion chromatography.

Non-denaturing PAGE and SDS-PAGE, and Western Blotting. Non-denaturing PAGE and SDS-PAGE were performed using 7.5, 10, or 10 to 20% gradient polyacrylamide Ready Gels from Bio-Rad (Hercules, CA). Protein bands were visualized with Coomassie Blue R (Sigma, St. Louis, MO) or electrotransferred onto a polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ) for Western blotting. The mouse AAIII-specific antibody MR-C1 and penta-His-specific antibody (Qiagen) were used at dilutions of 1:10,000. The mouse AAIII-specific antibody was used for Western blotting. Lanes 1 and 4) purified murine AAIII. An anti-mouse AAIII-specific rabbit polyclonal antibody MR-C1 was used for Western blotting. Lanes 1 and 4 were stained with Coomassie Blue R. Loading: lanes 1 and 2, ~40 μg; lanes 3 and 4, ~10 μg. B, size-exclusion chromatography of the purified murine untagged AAIII. Fractions containing the first (I) and second (II) peaks were cross-linked with bis(sulfosuccinimidyl) suberate, resolved on SDS-PAGE, and stained with Coomassie Blue R. Lane 1, the first peak; lane 2, the second peak. Loading: 40 μg (I) and 10 μg (II). Positions of size markers (GE Healthcare) in kilodaltons are shown on the left. C, electron micrograph of the tetrameric fraction of murine AAIII negatively stained with 1% uranyl acetate. The micrographs revealed predominant protein particles with close to 4-fold rotational symmetry.
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1:2000 and 1:5000, respectively. Secondary horseradish peroxidase-conjugated species-specific antibodies (Jackson Immunoresearch, West Grove, PA) were used at a dilution 1:20,000. Protein bands were visualized using an ECL kit and Hyperfilm ECL (GE Healthcare).

Size-Exclusion Chromatography. The separation of the AAIII tetramers from the AAIII dimers was performed on a 1 × 100 cm Sephacryl S-200 column (GE Healthcare) in phosphate-buffered saline. The column was calibrated with carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), lactate dehydrogenase (140 kDa), and catalase (232 kDa) (all from GE Healthcare). The major peak of 135 ± 10 kDa and a minor peak of 75 ± 10 kDa were detected (Fig. 1B), corresponding to the tetramer and dimer fractions (Pushkin et al., 2004). Freshly purified AAIII tetramer fractions not contaminated with AAIII dimers (Fig. 1) were used for kinetic studies.

Protein Cross-Linking. The AAIII dimer and tetramer fractions eluted from the Sephacryl S-200 column were cross-linked at 20°C for 30 min with 1 mM bis(sulfosuccinimidyl) suberate, obtained from Pierce (Rockford, IL), according to the manufacturer’s protocol. The cross-linking reaction was stopped with 0.2 M Tris-HCl, pH 7.5.

Electron Microscopy. Transmission electron microscopy was performed on a JEM-1200EX electron microscope (JEOL, Tokyo, Japan) at 80 kV and magnification of 75,000 ×. The enzyme was negatively stained with 1% uranyl acetate using a droplet technique (Fig. 1C). A Bioscan 600W digital camera (Gatan, Pleasanton, CA) was used to record the images.

AAIII Assay. AAIII activity was determined with different N-acetylated amino acids and mercapturic acids by measuring the amount of a deacetylated product in a fluorescence assay (Udenfriend et al., 1972). The reaction mixture, in a total volume of 0.1 ml, contained 50 mM Na-phosphate buffer, pH 7.5, a substrate and the enzyme. After incubation at 37°C for 30 min, the reaction was stopped by adding trichloroacetic acid to a final concentration 5%, the mixture was centrifuged at 12,000 g for 5 min, and 0.9 ml of 50 mM Na-phosphate was added to the supernatant. After adding 75 μl of fluorescein (Sigma) solution (10 mg/33 ml of acetone), fluorescence measurements (390 nm excitation, 475 nm emission) were performed on an LS-5 fluorescence spectrophotometer (PerkinElmer, Boston, MA). A calibration with methylamine was used for all substrates except N-acetyl-l-tryptophan, which was calibrated with l-tryptophan. Experiments were performed at least in triplicate except with N-acetyl-S-(4-bromomethyl)-L-cysteine.

The pH effect on mouse AAIII was studied using a 2 mM substrate concentration. The effect of p-chloromercuribenzoate (pCMB), dithiothreitol (DTT), EDTA, and metal ions on the activity of AAIII was studied with 2 mM N-acetyl-S-benzyl-L-cysteine. Incubations were performed at 37°C for 30 min. The effect of temperature on the activity of AAIII was studied with 2 mM N-acetyl-S-benzyl-L-cysteine; incubation time was 30 min.

The Km and Vmax were calculated by fitting the data to the Michaelis-Menten equation using GraphPad Prism software (GraphPad Software, San Diego, CA) or EZ-Fit software (Perrella Scientific, Amherst, NH).

Synthesis of Mercapturic Acids. NA-1,2-DCVC, NA-2,2-DCVC, N-acetyl-S-(1,2-trichlorovinyl)-l-cysteine, N-acetyl-S-(1,2,3,4-tetrafluoroethyl)-l-cysteine, N-acetyl-S-(2-chloro-1,2,2-trifluoroethyl)-l-cysteine, N-acetyl-S-(2,2-dichloro-1,1-difluoroethyl)-l-cysteine, N-acetyl-S-(2,2-dichloro-1,1-difluoroethyl)-l-cysteine, N-acetyl-S-(2,2-dichloro-1,1-difluoroethyl)-l-cysteine, N-acetyl-S-(2,2-dichloro-1,1-difluoroethyl)-l-cysteine, N-acetyl-S-(2-chloro-1,1-difluoroethyl)-l-cysteine, N-acetyl-S-(3-fluorobenzyl)-l-cysteine, N-acetyl-S-(4-chlorobenzyl)-l-cysteine, N-acetyl-S-(4-bromobenzyl)-l-cysteine, and N-acetyl-S-(4-methoxybenzyl)-l-cysteine were synthesized and characterized as described before (Birner et al., 1997; Werner et al., 1997; Scholz et al., 2005). N-Acetyl-l-tyrosine, N-acetyl-l-phenylalanine, N-acetyl-l-tryptophan, N-acetyl-l-histidine, N-acetyl-l-cysteine, N-acetyl-l-aspartic acid, N-acetyl-l-lysine, and N-acetyl-l-lysine were purchased from Sigma.

Cytotoxicity Measurements. Cytotoxicity measurements were performed using mPCT cells derived from mouse proximal tubule cells (Gross et al., 2001). The extracts from mPCT cells did not deacetylate NA-1,2-DCVC and NA-2,2-DCVC (data not shown), and therefore, these cells were used in our experiments to study the effect of the expression of mouse AAIII on the cytotoxicity of NA-1,2-DCVC and NA-2,2-DCVC. To express AAIII in mPCT cells, the coding sequence of mouse AAIII was inserted into the multiple cloning site of a pcDNA3.1 (+) Zeocin mammalian expression vector (Invitrogen), which contains the cytomegalovirus immediate-early promoter/enhancer for efficient high-level expression and the Zeocin resistance gene for selection.

All plasmids were purified with the Endofree plasmid purification kit (QIAGEN) before their use. mPCT cells were transfected with the plasmid using LipofectAMINE 2000 (Invitrogen) per the manufacturer’s protocol. Mock-transfected mPCT cells were generated by transfecting the cells with the vector. For cytotoxicity experiments, mPCT cells were used 24 h after transfection. The cells (0.5–10 × 106 cells/ml) were seeded into 96-well plates with Dulbecco’s modified Eagle’s medium (Invitrogen) containing 5% fetal bovine serum and different concentrations of NA-1,2-DCVC or NA-2,2-DCVC for 1 to 3 h at 32°C. These cells, incubated with the medium without mercapturic acids, were used as a control. In addition, mock-transfected cells incubated with and without mercapturic acids were used as additional controls. Cell viability was estimated using a CytoTox96 cytotoxicity assay (Promega, Madison, WI). The assay measures lactate dehydrogenase (LDH) activity released into the medium because of cell death. Cell media (50 μl) were mixed with 50 μl of the reconstituted substrate mix and incubated at 20°C for 30 min. The reaction was stopped by adding 50 μl of the stop solution. The absorbance of the solution at 490 nm (OD490) was measured on a VMax Kinetic Microplate Reader ( Molecular Devices, Sunnyvale, CA). The percentage of cytotoxicity was calculated by dividing experimental LDH release (OD490) by maximum LDH release (OD490). The maximum LDH release (equal to the total amount of cells) was accessed by lysing the cells with 1% Triton X-100 and LDH activity measurement in the lysate.

Data Analysis. All values are means ± S.E. of measurements of at least three separate experiments unless specifically indicated. Statistical significance of differences was determined by the unpaired Student’s t-test. A probability (p) level of <0.05 was considered as significant.

Results

Kinetic characteristics of murine AAIII are summarized in Table 1. The Km value was in the range of 0.25 mM for N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-l-cysteine to 5.3 mM for N-acetyl-S-(2,2-dichloro-1,1-difluoroethyl)-l-cysteine.

N-Acetyl-l-cysteine (substrate of AAIII) and N-acetyl-l-aspartic acid (substrate of AAII) were not deacetylated by murine AAIII. N-acetylated l-aromatic amino acids including tyrosine, phenylalanine, and tryptophane were good substrates of murine AAIII. The enzyme also deacetylated N-acetyl-l-histidine and N-acetyl-l-methionine (Pushkin et al., 2004). Mouse AAIII deacetylated N%-acetyl-l-lysine but not N%-acetyl-l-lysine, the substrate of N%-acetyl-l-lysine amidohydrolase.

The enzyme deacetylated N-acetyl-S-benzyl-l-cysteine. Fluorination of the aromatic ring significantly decreased Vmax of murine AAIII, whereas chlorination did not have significant effect on Vmax and bromination even increased it. Contrary to the nature of the introduced halogen, its position did not significantly affect Vmax.

Mouse AAIII possesses higher Vmax for mercapturic acids than for N-acetyl-l-amino acids except N-acetyl-S-(2,2-difluoro-1,1-dichloroethyl)-l-cysteine and fluoro derivatives of N-acetyl-S-benzyl-l-cysteine. Conversely, mouse AAIII had the highest Vmax with N-acetyl-S-(2,2-dichloro-1,1-difluoroethyl)-l-cysteine. The enzyme deacetylated the mercapturic acids derived from the TCE metabolism, NA-1,2-DCVC and NA-2,2-DCVC, and also N-acetyl-S-(1,2,2-trichlorovinyl)-N-acetyl-l-cysteine. The specific activity of the enzyme with NA-2,2-DCVC and N-acetyl-S-(1,2,2-trichlorovinyl)-l-cysteine was higher than that with NA-1,2-DCVC.

Comparison of Vmax/Km values suggested that NA-2,2-DCVC is the most efficient and N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-l-cysteine is the least efficient substrate of murine AAIII. Substitution of fluorine at carbon 2 of the S-(2-chloro-1,1,2-trifluoroethyl) group in N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-l-cysteine with chlorine significantly increased the Vmax/Km value mainly because of a big rise of Vmax, which was significantly higher than the increase of Km.
Mouse AAIII used in the present study possessed tetrameric structure (Fig. 1), suggesting potential allosteric interactions that might lead to deviations of substrate saturation curves from the hyperbolic shape. Nevertheless, no deviations from the Michaelis kinetics were detected for mouse AAIII in the present or previous study (Pushkin et al., 2004).

Mouse AAIII had a pH optimum in 50 mM Na-phosphate buffer with the substrates studied in the present article, in the range of pH 7.5 to 7.7. The temperature optimum of murine AAIII with most of the substrates used was ~40°C. The activity of the enzyme was not affected by EDTA and, in addition, by Zn²⁺, Mg²⁺, and Ca²⁺ (up to 30 mM), indicating that the murine AAIII-mediated catalysis does not require metal ions.

DTT significantly activated (Fig. 2A) and pCMB inhibited (Fig. 2B) murine AAIII, indicating that the cysteine residues are important for the enzyme function. There are eight cysteines in murine AAIII, whereas rat AAIII and human AAIII have seven and five cysteine residues, respectively (Fig. 2C). Four mouse AAIII cysteines (Cys⁶¹, Cys¹⁵⁵, Cys¹⁶⁵, and Cys¹⁸⁵) are common in all three AAIIIs (Fig. 2C) and therefore may be important for the catalysis.

AAIII was shown in this study to deacetylate several mercapturic acids. We hypothesized that the deacetylation of mercapturic acids mediated by AAIII may be involved in their cytotoxicity. To explore this hypothesis, the cytotoxicity of two TCE metabolites shown, NA-1,2-DCVC and NA-2,2-DCVC, was studied. Mouse proximal tubule-derived mPCT cells, which do not endogenously express AAIII protein, were used in these experiments (Fig. 3A). In addition, no NA-1,2-DCVC- or NA-2,2-DCVC-deacetylation activity was detected in the extracts from the untransfected mPCT cells, whereas the extracts from the mPCT cells expressing AAIII deacetylated both mercapturic acids (data not shown). The expression of mouse AAIII, confirmed by Western blotting (Fig. 3A), significantly increased the toxicity of NA-1,2-DCVC in comparison with the untransfected or mock-transfected mPCT cells (Fig. 3, B and C), whereas the better AAIII substrate, NA-2,2-DCVC, was less toxic. An inhibitor of cysteine S-conjugate β-lyases, aminooxyacetate (0.2 mM), significantly decreased the toxic effects of NA-1,2-DCVC and NA-2,2-DCVC. These data suggested that AAIII mediates deacetylation of the mercapturic acids derived from the metabolism of TCE in the proximal tubule cells, and this process is involved in their toxicity. Absence of a strict correlation between the Vₘₐₓ of AAIII for the mercapturic acid and its cytotoxicity indicated that, in addition to the AAIII activity, other cellular factors are involved in mediating their cytotoxicity.

**Discussion**

This article provides new insights into the substrate specificity of AAIII in comparison with other aminoacylases. Until our work, there was limited data regarding substrates that can be used by AAIII (Pushkin et al., 2004). Although previous studies have characterized partially purified aminoacylases from rat liver (Suzuki and Tateishi, 1981) and rat kidney (Endo, 1978; Uttamsingh and Anders, 1999), with substrate specificities similar to those of AAIII, their potential contamination with other aminoacylases and effectors of AAIII could significantly affect these results.

Mouse AAIII deacetylated N-acetyl-L-tyrosine, N-acetyl-L-phenylalanine, N-acetyl-L-tryptophan, and N-acetyl-L-histidine in a manner similar to that of partially purified rat kidney acylase (Suzuki and Tateishi, 1981) and rat kidney (Endo, 1978; Uttamsingh and Anders, 1999), with substrate specificities similar to those of AAIII, their potential contamination with other aminoacylases and effectors of AAIII could significantly affect these results.

Mouse AAIII deacetylated N-acetyl-L-tyrosine, N-acetyl-L-phenylalanine, N-acetyl-L-tryptophan, and N-acetyl-L-histidine in a manner similar to that of partially purified rat kidney acylase (Endo, 1978); but, contrary to rat kidney acylase, they did not deacetylate N-acetyl-L-aspartic acid, the substrate of AAIII. Mouse AAIII also deacetylated N⁴-acetyl-L-lysine but not N⁴-acetyl-L-lysine, indicating that the presence of the free carboxyl group in close proximity to the amino group is important for the substrate recognition of AAIII. N-Acetyl-L-cysteine, a substrate of purified rat kidney AAI (Uttamsingh et al., 1998), was not used by mouse AAIII (Pushkin et al., 2004). N-Acetyl-L-methionine was deacetylated by both mouse AAIII (Pushkin et al., 2004) and rat kidney AAII (Uttamsingh et al., 1998).

Mouse AAIII was shown in the present study to deacetylate several halogenated mercapturic acids, metabolic products of environmental toxicants, including N-acetyl-S-(2,2-dichloro-1,1-difluoroethyl)-L-cysteine, N-acetyl-S-(1,2,2-trichlorovinyl)-L-cysteine, NA-1,2-DCVC, NA-2,2-DCVC, and N-acetyl-S-(1,2,3,4,4-pentachlorobutadienyl)-L-
cysteine. Several halogenated mercapturic acids including N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine and N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine were also deacetylated by AAI (Uttamsingh et al., 1998). The specific activity of AAI was maximal with N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine. It decreased with the substitution of fluoride for chloride or bromide, suggesting that the increase of molar volume of a mercapturate decreases AAI activity (Uttamsingh et al., 1998). Our data indicate that the substitution of two fluoride groups in N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine for two chloride groups makes it a good substrate of AAIII if at least one of the substituted fluoride groups is located at carbon 2 of the S-(tetrahaloethyl) group. Therefore, in addition to the nature of halogen atom in the S-(tetrahaloethyl) group, its position is very important for the $V_{max}$ of AAIII. For example, N-acetyl-S-(2,2-dichloro-1,1-difluoroethyl)-L-cysteine has an $\sim$30 times higher deacetylation rate than N-acetyl-S-(2,2-difluoro-1,1-dichloroethyl)-L-cysteine. It is not known whether N-acetyl-S-(2,2-fluoro-1,1-dichloroethyl)-L-cysteine is a substrate of AAI. But even if this is so, it
seems unlikely that it is a good substrate of AAII since the $V_{\text{max}}$ of AAII significantly decreased by replacement of even one fluoride group for chloride at carbon 2 of the $S$-(1,1,2,2-tetrafluoroethyl) group in N-acetyl-$S$-(1,1,2,2-tetrafluoroethyl)-l-cysteine. Figure 4 summarizes differences in the substrate specificity of AAIII and AAII toward certain mercapturates.

AAIII was shown to deacetylate N-acetyl aromatic l-αmino acids and N-acetyl-S-benzyl-L-cysteine (Anders and Dekant, 1994; Uttam-singh and Anders, 1999; Pushkin et al., 2004). Mouse AAIII had higher affinity to N-acetyl-S-benzyl-l-cysteine than did rat kidney acylase (Uttamsingh and Anders, 1999). In the present article, we demonstrated that the halogenation of the aromatic ring in N-acetyl-S-benzyl-l-cysteine changed the $V_{\text{max}}$ value of mouse AAIII: fluorination significantly decreased $V_{\text{max}}$, whereas bromination increased it; chlorination did not have significant effect on $V_{\text{max}}$. Halogenation of several substrates of rat AAIII was also shown to increase its specific activity (Uttam-singh et al., 1998), but contrary to AAIII, the highest $V_{\text{max}}$ was detected with fluoro derivatives and the lowest with bromo derivatives. In the present study, we analyzed how single substitution of hydrogen atoms in the aromatic ring of N-acetyl-S-benzyl-l-cysteine for halogen atoms affects the enzyme $V_{\text{max}}$ and $K_m$ values. The results indicated (Fig. 4) that the substitutions for chloride and bromide groups did not significantly change $V_{\text{max}}$, whereas substitutions for fluoro or methoxy groups significantly decreased it. Therefore, similar to S-alkyl-N-acetylcysteines, the substitutions of the aromatic ring in N-acetyl-S-benzyl-l-cysteine for fluorine in general decrease the $V_{\text{max}}$ of AAIII, whereas the substitutions for chlorine and bromine increase the $V_{\text{max}}$ of AAIII. The effect of substitutions of hydrogen atoms for halogen atoms in the benzyl group of N-acetyl-S-benzyl-l-cysteine on $K_m$ of AAIII depends more on the position than on the nature of the halogen atom (Table 1).

Mouse AAIII studied in this article possessed tetrameric structure. Nevertheless, no deviations from the Michaelis kinetics were detected in our study with any substrates, indicating that there were no allosteric interactions between the monomers of AAIII. The data suggest that either there are no allosteric interactions between the putative AAIII monomer active sites, or AAIII tetramer has a single active site. Against the last hypothesis is the detection of an $\sim 55$-kDa rat kidney aminoacylase (potentially dimeric) with specificity similar to that of AAIII (Endo, 1978; Uttamsingh and Anders, 1998) and mouse AAIII dimers in the present work. In addition to determining the enzymatic activity of the purified AAIII dimer, it is also necessary to determine whether a single AAIII monomer possesses the enzymatic activity. It should be mentioned that AAII, known to form a homodimer (Kordel and Schneider, 1976; D’Ambrosio et al., 2003; Lindner et al., 2003), also demonstrates Michaelis kinetics (Uttamsingh et al., 1998; Lind-\textunderscore{ner} et al., 2005).

The pH optimum of mouse AAIII (~7.5–7.7) is close to the pH optimum of rat liver acylase (Suzuki and Tateishi, 1981) and rat kidney acylase (Endo, 1978). It is also close to the pH optima of AAII and AAII (Anders and Dekant, 1994). Contrary to AAII and similar to AAII (Anders and Dekant, 1994), the AAIII-mediated catalysis does not require metal ions.

Activation by DTT and inhibition by pCMB of murine AAII suggested that cysteine residues are important for the enzyme function. Rat liver acylase, with a substrate specificity similar to that of AAIII, was also inhibited by pCMB (Suzuki and Tateishi, 1981).

$p$-Hydroxymercuribenzoate also inhibited AAII (D’Adamo et al., 1977), whereas DTT was shown to activate or inhibit AAII isolated from different sources (Matsumoto and Nagai, 1972; Anders and Dekant, 1994). Determination of whether cysteine residues play a structural role and/or are involved in the AAIII-mediated catalysis requires further studies.

Comparison of the substrate specificity of AAIII with AAII and AAII indicates that AAIII and AAII use a broad spectrum of substrates, whereas only N-acetyl l-aspartic acid is efficiently used by AAII. Nevertheless, AAIII has much higher sequence homology with AAII, but not with AAII (Pushkin et al., 2004). This may indicate that the catalytic mechanisms mediated by AAIII and AAII are similar, although the substrate recognition sites are different.

The mercapturic acids studied in this work were, in general, good substrates of AAIII. Comparison of two isomers, NA-1,2-DCCV and NA-2,2-DCCV, indicates that the latter is a better substrate of murine AAIII (Table 1). Therefore, in cells expressing AAIII, NA-2,2-DCCV is expected to be more toxic than NA-1,2-DCCV if the cytotoxicity is determined by the AAIII-mediated deacetylation rate only. Conversely, current data indicate that, instead, NA-1,2-DCCV is significantly more nephrotoxic than NA-2,2-DCCV (Birner et al., 1997) and significantly more cyto-toxic in proximal tubule cells (Commandeur et al., 1991; Anders and Dekant, 1998). In our experiments, in which, contrary to previous studies, AAIII was endogenously expressed in mouse proximal tubule mPCT cells, NA-1,2-DCCV was more toxic at high dose (0.75 mM) than was NA-2,2-DCCV. The toxic effect was significantly inhibited by aminooxyacetate, suggesting that the toxicity of these mercapturic acids is mediated by $\beta$-lyase. The higher cytotoxicity of NA-1,2-DCCV than of NA-2,2-DCCV in our experiments suggested that mPCT cells have higher N-acetyltransferase activity toward 2,2-DCCV than toward 1,2-DCCV, which compensated for the higher rate of NA-2,2-DCCV deacetylation compared with NA-1,2-DCCV. In agreement with this hypothesis, the activity of N-acetyltransferase in kidney microsomal membranes isolated from Wistar rats was shown to be a few times higher with NA-2,2-DCCV than with NA-1,2-DCCV (Birner et al., 1997). Assuming that similar dependence exists in mouse, one can suggest that at constant levels of AAIII and N-acetyltransferase, and the same mercapturate dose, the 2,2-DCCV/NA-2,2-DCCV ratio is significantly smaller than the 1,2-DCCV/NA-1,2-DCCV ratio. The cytotoxicity of NA-1,2-DCCV toward isolated rat kidney proximal tubular cells demonstrated a delayed time course compared with 1,2-DCCV, whereas the time courses of cytotoxicity of NA-2,2-DCCV and 2,2-DCCV were parallel (Commandeur et al., 1991), which is compatible with our data by which 2,2-DCCV was shown to be a better substrate of AAIII. In addition, as was suggested before (Birner et al., 1997), the lower nephrotoxicity of NA-2,2-DCCV may be related to the smaller rate of the $\beta$-lyase-dependent bioactivation of 2,2-DCCV compared with 1,2-DCCV (Commandeur et al., 1991).

Rabbit, rat, and guinea pig were shown to deacytelylate in vivo several mercapturic acids including N-acetyl-S-benzyl-l-cysteine, N-acetyl-S-butyl-l-cysteine, and N-acetyl-S-(2-chloro-4-nitrophenyl)-l-cysteine (Bray and James, 1960). Rat renal proximal tubular cells deacetylated N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-l-cysteine, N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-l-cysteine, N-acetyl-S-(2,2-dichloro-1,1-difluoroethyl)-l-cysteine, and N-acetyl-S-(2,2-dibromo-1,1-difluoroethyl)-l-cysteine (Boogaard et al., 1989). Rat kidney cysteol deacytelylated N-acetyl-S-pentachlorobutadienyl-l-cysteine, N-acetyl-triclorovinyl-l-cysteine, and NA-1,2-DCCV (Vamvakas et al., 1987; Pratt and Lock, 1988). Our data highlight those of the mercapturic acids, for which AAIII plays a key role in deacytelylation and suggest that the process is involved in their cytotoxicity.
FIG. 4. A, halogenated N-acetyl-S-ethyl-L-cysteines used as substrates by AAI and AAIII. (I): N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine; (II): N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine; (III): N-acetyl-S-(2-bromo-1,1,2-trifluoroethyl)-L-cysteine; (IV): N-acetyl-S-(2,2-difluoro-1,1-dichloroethyl)-L-cysteine; (V): N-acetyl-S-(2,2-dichloro-1,1-difluoroethyl)-L-cysteine; and (VI): N-acetyl-S-(2,2-difluoro-1,1-dichloroethyl)-L-cysteine. B, halogenated N-acetyl-S-vinyl-L-cysteines used as substrates by AAIII. (VII): N-acetyl-S-(1,2,3,4,4-pentachlorobutenyl)-L-cysteine; (VIII): N-acetyl-S-(1,2,2-trichlorovinyl)-L-cysteine; (IX): NA-2,2-DCVC; and (X): NA-2,2-DCVC. C, halogenated N-acetyl-S-benzyl-L-cysteines used as substrates by AAIII. (NASBC): N-acetyl-S-benzyl-L-cysteine; (NA2FBC): N-acetyl-S-(2-fluorobenzyl)-L-cysteine; (NA2CBC): N-acetyl-S-(2-chlorobenzyl)-L-cysteine; (NA3FBC): N-acetyl-S-(3-fluorobenzyl)-L-cysteine; (NA4CBC): N-acetyl-S-(4-chlorobenzyl)-L-cysteine; (NA4BBC): N-acetyl-S-(4-bromobenzyl)-L-cysteine; and (NA4MBC): N-acetyl-S-(4-methoxybenzyl)-L-cysteine. White columns show \( V_{\text{max}} \) (μmol·mg⁻¹·min⁻¹), black columns show \( K_m \) (mM), and gray columns show \( V_{\text{max}}/K_m \) (mL·mg⁻¹·min⁻¹).