Structural characterization, tissue distribution, and functional expression of murine aminoacylase III

Alexander Pushkin, Gerardo Carpenito, Natalia Abuladze, Debra Newman, Vladimir Tstrupun, Sergey Raizantsev, Srilakshmi Motemoturu, Pakan Sassani, Nadezhda Solovieva, Ramnath Dukkipati, and Ira Kurtz

Division of Nephrology, David Geffen School of Medicine, University of California at Los Angeles, California 90095; Department of Otolaryngology, University of Minnesota, Minneapolis, Minnesota 55455; and Department of Biological Chemistry, David Geffen School of Medicine, University of California at Los Angeles, California 90095

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Many toxic substances can be eliminated from the body after transformation to N-acetyl-L-cysteine S-conjugates or mercapturic acids (4, 6, 9, 45). The synthesis of mercapturic acids involves at least four enzymatic reactions (19, 35) that are catalyzed by glutathione S-transferase, γ-glutamyltranspeptidase, dipeptidase, and N-acetyltransferase. The major sites of mercapturic acid synthesis are liver and kidney. Mercapturic acids synthesized in liver may be transported either across the canalicular membrane into bile (19) or to the kidney, which is the major route for their elimination (20, 35). These excretory pathways play an important role in preventing nephrotoxicity (9, 11, 35). Kidneys and especially renal proximal tubules are most sensitive to the toxic cysteine S-conjugates (2, 7, 10, 21, 26).

In kidneys, mercapturic acids can be secreted into urine by proximal tubules (2, 7, 10, 21, 26). Localized to the basolateral membrane of S1, S2, and S3 proximal tubules (25, 39), the organic anion transporter 1 (OAT1) was recently shown to mediate the cellular influx of mercapturic acids into proximal tubule cells (34). The organic acid transporter OAT3 has overlapping substrate specificity and is probably involved in this process as well (5). Masereu et al. (31) have shown that mercapturic acids can be secreted through the brush-border membrane into urine using the multidrug-resistance-associated protein (MRP2) and/or diffusion.

Mercapturic acids may become nephrotoxic in a sequence of reactions catalyzed by aminoacylase and β-lyase. In general, the product of the deacetylation reaction is not toxic; however, it becomes toxic in a subsequent reaction that is catalyzed by cysteine S-conjugate β-lyase (2, 7–10, 23, 24, 38, 40), which is localized to mitochondria. Mitochondria are especially vulnerable to toxic cysteine S-conjugates (17, 32). However, despite the fact that the mitochondrial mass is greatest in S1 tubules (12), many cysteine S-conjugates are more toxic for S2 and S3 proximal tubule segments (22, 23, 32, 46). The variation among proximal tubule segments in sensitivity to cysteine S-conjugates may be due to differences in the intracellular distribution and characteristics of β-lyase and aminoacylase among proximal tubule segments. β-Lyase is uniformly expressed in all three segments of proximal tubule and is therefore not likely responsible for the variation in toxicity (23, 24, 30). There are two aminoacylases that could contribute to the nephrotoxicity to cysteine S-conjugates. Aminoacylase I (EC 3.5.1.14, N-acetylamino acid hydrolyase) has been shown to catalyze the deacetylation of neutral aliphatic N-acetyl-α-amino acids including N-acetyl-l-cysteine, 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-bromo-1,1,2-trifluoroethyl)-l-cysteine, and several S-alkyl-N-acetylcysteines (1, 16, 18, 27, 28, 42, 43). However, the aminoacylase I transcript, similar to β-lyase, is uniformly expressed throughout proximal tubules (29). Therefore, aminoacylase I is unlikely responsible for the variation in toxicity among proximal tubule segments.

Address for reprint requests and other correspondence: A. Pushkin, UCLA Division of Nephrology, 10833 Le Conte Ave., Rm. 7-155 Factor Bldg., Los Angeles, CA 90095-1689 (E-mail: apushkin@mednet.ucla.edu).
An aminoacylase that preferentially catalyzes the deacetylation of N-acetylatedaromatic amino acids has been partially purified from rat liver cytosol (36) and rat kidney (13, 42). In addition, this partially purified enzyme catalyzes N-deacetylation of some S-aryl- and S-aralkylmercaptopurines including N-acetyl-L-(1,2-dichlorovinyl)-L-cysteine, N-acetyl-L-(2,2-dibromo-1,1-difluoroethyl)-L-cysteine, N-acetyl-L-S-(1,2,3,4,4-pentaclorobutadienyl)-L-cysteine, and N-acetyl-S-benzyl-L-cysteine, and is therefore also a potential candidate for mediation of the nephrotoxicity of some mercapturates. However, the molecular identity of this aminoacylase is currently unknown. Given its potential role in mediating the nephrotoxicity of mercapturates and the deacetylation of N-acetyl-S-aromatic amino acids, a goal of the present study was to clone the enzyme, characterize its functional properties, and determine its oligomeric structure and localization in kidney.

MATERIALS AND METHODS

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

Cloning of mouse kidney aminoacylase III. The coding sequence for aminoacylase III was identified by searching the mouse Expressed Sequence Tag database (dbEST) for homology with mouse aminoacylases I and II. Several overlapping dbEST clones were identified in the house mouse (Mus musculus) database with homology to aminoacylase II. We identified an IMAGE clone (4155233, Invitrogen, San Diego, CA) that contained the complete open reading frame of aminoacylase III. To confirm that the mouse aminoacylase III sequence was derived from a bona fide transcript, we amplified the entire open reading frame via PCR using mouse kidney cDNA (Clontech, Palo Alto, CA). Nucleotide sequences were determined bidirectionally using automated sequencing (ABI 310, Perkin Elmer, Foster City, CA).

Northern analysis. Northern blots with various mouse tissues were obtained from Origene (Rockville, MD). The probe was random prime labeled with [32P]dCTP to a specific activity of ~1.5 × 106 dpm/μg. The filters were prehybridized at 42°C for 2 h using 50% formamide, 6× standard saline phosphate EDTA (SSPE), 0.5% SDS, Denhardt’s solution, and 0.1 mg/ml sheared herring testes denatured DNA. After the prehybridization, filters were incubated with the 32P probe using 25 ml of hybridization buffer. The probe was denatured and added to the hybridization solution at 107 dpm/ml. The filters were probed at 42°C for 18 h and washed in 1× SSC (0.15 M NaCl in 15 mM sodium citrate), 0.1% SDS at 45°C for 60 min (4 changes, 400 ml/wash) and were exposed on BioMax MS film (Eastman Kodak, Rochester, NY). The probe used to identify mouse aminoacylase III was a 226-bp PCR product. The following primers were used: sense, 5'-GGGGCACAGTCTATTTTATATAATGAGAT-3'; antisense, 5'-GGGACACGTCATTTTTATATAATGAGAT-3'.

Generation and characterization of polyclonal antibodies to mouse aminoacylase III. A polyclonal antibody to mouse aminoacylase III (MR-C1) was raised in rabbits against a synthetic peptide derived from the COOH terminus of the protein corresponding to amino acids 302–317 in mouse aminoacylase III coupled to an NH2-terminal cysteine. The antibody was affinity purified using Sepharose 4B columns with a covalently attached aminoacylase III peptide. This antibody identified an ~35-kDa band upon SDS-PAGE of mouse kidney homogenates and mouse kidney microsomal membranes, and an ~38-kDa band in extracts from human embryonic kidney (HEK)-293 cells that expressed His-tagged mouse aminoacylase III (Fig. 1). Penta-His antibody from Qiagen (Valencia, CA) yielded an identical size for the His-tagged fusion aminoacylase III that was expressed in HEK-293 cells (Fig. 1).

Purification of mouse aminoacylase III from HEK-293 cells. The coding region of aminoacylase III was inserted into the BamHII-XhoI site of a pcDNA3.1-His vector (Invitrogen, Carlsbad, CA). The construct was expressed as a His6-tagged fusion protein in HEK-293 cells. The cells (~1 g) were collected 24 h after transfection, washed three times with PBS (10 mM sodium phosphate, pH 7.4), and suspended in 20 ml of 50 mM Tris-HCl buffer that contained 1% Triton X-100 and the following protease inhibitors: 1 mM PMSF, 1 mM EDTA, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μg/ml aprotinin (all protease inhibitors were from Roche, Indianapolis, IN). After the homogenate was incubated for 30 min, it was centrifuged at 18,000 × g for 20 min at 4°C. The supernatant was dialyzed for 16 h at 4°C against PBS and purified on a Ni-superflow resin (Novagen, Madison, WI) column (2 × 8 cm) according to the manufacturer’s protocol. The fractions that contained His6-tagged aminoacylase III were combined, dialyzed against 20 mM Tris-HCl, pH 7.5, and loaded into a 3 × 6-cm column of DEAE-cellulose DE52 (Whatman, Maidstone, Kent, UK) equilibrated with the same buffer. The proteins were eluted onto a 0–250 mM

Fig. 1. A: characterization of the anti-mouse aminoacylase III-specific polyclonal antibody MR-C1. Mouse proteins (lanes 1 and 2) and extracts from human embryonic kidney (HEK)-293 cells transfected with pcDNA3.1-His vector containing the coding sequence of mouse aminoacylase III (lanes 3 and 4) and from untransfected cells (lane 5) were incubated with MR-C1 antibody (lanes 1, 3, and 5) or with the antibody preincubated with the immunizing peptide (10 μg/ml, lanes 2 and 4). B: characterization of the His6-tagged mouse aminoacylase III fusion protein purified from HEK-293 cells: ~4 μg of the purified protein was separated using SDS-PAGE and was stained with Coomassie blue R (lane 1), or 5 ng was transferred onto PVDF membrane and probed with the MR-C1 antibody (lane 2), with MR-C1 antibody preincubated with the immunizing peptide (lane 3) or with penta-His antibody (lane 4). His-tagged aminoacylase III was cleaved with enterokinase and probed with MR-C1 (lane 5) and penta-His antibody (lane 6). C: Western blot of the cytosol (lanes 1 and 3) and microsomal membranes (lanes 2 and 4) isolated from mouse kidney. Loading: 80 μg (lanes 2 and 4) and 8 μg (lanes 1 and 2). MR-C1 antibody (lanes 1 and 2) or MR-C1 antibody preincubated with the immunizing peptide (10 μg/ml, lanes 3 and 4) was used. Positions of size markers are shown (in kDa; left).
Tris-HCl gradient. Fractions that contained aminocaylase III were combined and then concentrated and transferred to PBS using a Centriplus YM-10 centrifugal filter device (Millipore, Bedford, MA). All purification procedures were performed at 4°C. SDS-PAGE and Western blotting (Fig. 1) showed one protein band that reacted with both mouse kidney aminocaylase III–specific MR-C1 and penta-His antibodies. The size of the purified fusion protein was ∼38 kDa, which is in good agreement with the size predicted based on the amino acid composition for mouse kidney aminocaylase III (∼35 kDa) plus ∼3 kDa for the His6 tag.

The His6-containing vector sequence of ∼3 kDa fused with the NH2 terminus of mouse kidney aminocaylase III could potentially affect the kinetic characteristics and the oligomeric structure of the enzyme. Therefore the fusion protein was treated with a recombinant enterokinase (Novagen) to hydrolyze the enterokinase site between the vector and the NH2-terminal coding region of the enzyme. After digestion was completed (as determined by SDS-PAGE and Western blotting (Fig. 1) it was used in all experiments.

**Purification of aminocaylase III from mouse kidney.** Mouse kidneys (∼1 g) were disrupted in a glass homogenizer in 50 ml of 50 mM Tris-HCl, pH 7.5, that contained 1% Triton X-100 and the following protease inhibitors: 1 mM PMSF, 1 mM EDTA, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. After 45 min of incubation, the homogenate was centrifuged at 18,000 g for 20 min at 4°C. The supernatant was dialyzed overnight against 50 mM Tris-HCl, pH 7.5, that contained the protease inhibitors and was loaded onto a DEAE-cellulose column equilibrated with the same buffer. Proteins were eluted onto a 0–0.3 M linear gradient of NaCl in 50 mM Tris-HCl, pH 7.5. Fractions that contained aminocaylase III (detected by Western blotting) were selected on a Microcon 10 filter device (Amicon, Beverly, MA) and used for the molecular mass determination of aminocaylase III via size-exclusion chromatography.

**Microsomal membrane isolation from mouse kidney.** Mouse kidneys (∼2 g) were disrupted in a glass homogenizer in 100 ml of 50 mM Tris-HCl, pH 7.5, that contained 5% sucrose and the following protease inhibitors: 1 mM PMSF, 1 mM EDTA, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. The homogenate was centrifuged at 300 g for 10 min, and the supernatant was centrifuged at 4,000 g for 10 min and then at 18,000 g for 15 min. The resulting supernatant was centrifuged at 105,000 g for 2 h. The pellet was suspended in the buffer used for homogenization and centrifuged at 105,000 g for 1 h. This step was repeated twice. The final pellet was analyzed by SDS-PAGE and Western blotting using the aminocaylase III–specific antibody MR-C1.

**SDS-PAGE and Western blotting.** SDS-PAGE was performed using a 10–20% gradient of polyacrylamide Ready Gels (Bio-Rad, Hercules, CA). Proteins separated by SDS-PAGE were electrototransferred onto PVDF membrane (Amersham Biosciences, Piscataway, NJ). Nonspecific binding was blocked by incubation for 1 h in Tris-buffered saline (TBS: 20 mM Tris-HCl, pH 7.5, 140 mM NaCl) that contained 5% dry milk and 0.05% Tween 20 (Bio-Rad). The MR-C1 aminocaylase III–specific antibody and mouse penta-His antibody were used at dilutions of 1:2,000 and 1:5,000, respectively. Secondary horseradish peroxidase-conjugated species-specific antibodies (Jackson Immunoresearch, West Grove, PA) were used at a dilution of 1:20,000. Bands were visualized using an ECL kit and Hyperfilm ECL (Amersham Biosciences).

Aminocaylase III functional assay. Aminocaylase III activity was determined with different N-acetylated amino acids by measuring the amount of deacetylated product that was formed as determined with different aminocaylase II and mouse aminocaylase I, respectively. The enzyme has consensus sites (on the Internet, see http://www.expasy.org/cgi-bin/scanprosite) for N-glycosyla-
tion (Asn\textsuperscript{70} and Asn\textsuperscript{117}), tyrosine sulfation (Tyr\textsuperscript{88}, Tyr\textsuperscript{165}, and Tyr\textsuperscript{272}), protein kinase C (Thr\textsuperscript{298}), and casein kinase II (Thr\textsuperscript{83}, Ser\textsuperscript{130}, Ser\textsuperscript{160}, Thr\textsuperscript{201}, and Ser\textsuperscript{266}) phosphorylation, and myristylation (Gly\textsuperscript{18}, Gly\textsuperscript{19}, Gly\textsuperscript{22}, Gly\textsuperscript{27}, and Gly\textsuperscript{185}). The hydrophobicity analysis (on the Internet, see http://sosui.proteome.bio.tuat.ac.jp/sosui_submit.html) predicted that the enzyme is a cytoplasmic protein without transmembrane segments (data not shown). Aminoacylase III is encoded by mouse chromosome 19.

The expression of aminoacylase III in mouse tissues was studied using Northern blotting (Fig. 3). An \~1·4-kb transcript was detected that was expressed predominantly in kidney and to a lesser extent in liver. Transcripts were also detected at a significantly lower level in heart, small intestine, brain, lung, testis, and stomach. In testis, an additional transcript of \~2·2 kb was detected that was expressed equally with the \~1·4-kb transcript. Immunocytochemistry studies of mouse kidney indicated that the enzyme was localized predominantly in the apical membrane of cells in the S1 segment (Fig. 4). In subcellular fractionation experiments of mouse kidney, \~10\% of aminoacylase III was membrane associated (see Fig. 1C). In proximal straight tubules (S2 and S3 segments), the enzyme was expressed diffusely throughout the cytoplasm (Figs. 5 and 6).

SDS-PAGE and Western blotting of mouse kidney homogenates revealed an \~35-kDa band (see Fig. 1) of mouse kidney aminoacylase III. The untagged recombinant enzyme purified to homogeneity from HEK-293 cells had the same size (see Fig. 1). To determine the oligomeric structure of the enzyme, size-exclusion chromatography and transmission electron microscopy were used. Figure 7 illustrates a typical separation of

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**Fig. 2.** Alignment of mouse aminoacylases I, II, and III (GenBank accession nos. AK003703, AF212998, and AY169234, respectively).

**Fig. 3.** Northern blot analysis of aminoacylase III expression in mouse tissues. Multiple tissue blots were from Origene; blots were exposed on BioMax MS film from Eastman-Kodak. Tissues studied include brain (lane 1), heart (lane 2), kidney (lane 3), liver (lane 4), lung (lane 5), muscle (lane 6), skin (lane 7), small intestine (lane 8), spleen (lane 9), stomach (lane 10), testis (lane 11), and thymus (lane 12). A 226-bp mouse kidney aminoacylase III-specific probe was used. A: a 24-h exposure period. B: a 0.5-h exposure period. Positions of size markers are shown (in kb; left).
the untagged recombinant purified mouse aminoacylase III and aminoacylase III purified from mouse kidney on a Sephacryl S-200 column. In both cases, a single protein peak was detected that corresponded to a globular protein with molecular mass of \(110 \text{ kDa}\). The distribution of the enzymatic activity was identical to the distribution of the recombinant protein density, which indicates that this peak belongs to the enzyme. A comparison of this value with the minimum molecular mass determined by SDS-PAGE suggested that the enzyme is a homotetramer.

A tetrameric structure for the enzyme was further confirmed using transmission electron microscopy. Figure 8A illustrates a
typical electron micrograph of negatively stained molecules of
the untagged purified mouse kidney aminoacylase III. Square-
shaped molecular projections of \( \sim 9 \) nm were most frequently
seen. Representative molecular projections selected for image
processing are shown in Fig. 8B. A computer-averaged image
of these projections after reference-free alignment shows four
protein densities arranged with a fourfold rotational symmetry
(Fig. 8C), which suggests that the aminoacylase III molecule
consists of multiples of four monomers. However, the molecu-
lar mass of the enzyme determined by size-exclusion chro-
matography (130–140 kDa) and estimated molecular mass of
the monomer of mouse aminoacylase III of \( \sim 35 \) kDa (from
SDS-PAGE data and the amino acid composition) indicate that
a fourfold rotational symmetry is consistent only with a tet-
rameric structure of the molecule.

The kinetic characteristics of mouse aminoacylase III ex-
pressed in HEK-293 cells and purified to homogeneity are
shown in Table 1. The enzyme used as a substrate the N-
acetylated \( L \)-aromatic amino acids tyrosine, phenylalanine, and
tryptophan, similar to aminoacylase III partially purified from
rat kidney (13) and rat liver (36). The enzyme had higher
affinity to \( S \)-benzyl-N-acetylcyesteine than the purified rat kid-
ney aminoacylase (42). \( N \)-acetylcyesteine (a substrate of ami-
noacylase I) and \( N \)-acetylaspartate (a substrate of aminoacylase
II or aspartoacylase) were not hydrolyzed by mouse aminoacy-
lase III. Therefore, homogeneous recombinant mouse acylase
III has substrate specificity similar to partially purified amino-
acylases from rat kidney and liver. Mouse aminoacylase III has
a pH optimum (with \( S \)-benzyl-N-acetylcyesteine and \( N \)-acetyl-
tryosine) of \( \sim 7.5–7.7 \), which is similar to partially purified rat
liver and kidney aminoacylases (13, 36). Absence of signif-
icable kinetic differences between purified recombinant murine ami-
noacylase III expressed in HEK-293 cells and partially purified
aminoacylase from rat kidney (13) and rat liver (36) indicates

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Fig. 6. Immunolocalization of aminoacylase III in mouse kidney using MR-C1 antibody. a: immunofluorescence image depicts
cytoplasmic staining of S3 tubules. b: Nomarski image of the same field as in a. c: superimposed fluorescence from a and Nomarski
image from b. d: specific blocking of aminoacylase III staining with the immunizing peptide (10 \( \mu \)g/ml). e: Nomarski image
corresponding to d.

Fig. 7. Size-exclusion chromatography of mouse aminoacylase
III on a column (1 \( \times \) 80 cm) of Sephacryl S-200 equilibrated
with PBS. Column was calibrated with carbonic anhydrase (30
kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa),
lactate dehydrogenase (140 kDa), and catalase (232 kDa). A: untagged aminoacylase III purified from HEK-293 cells; \( \circ \),
protein concentration; \( \bullet \), aminoacylase III activity. B: untagged aminoacylase III purified from mouse kidney; o,
relative amount of the enzyme as determined by Western blotting; \( \bullet \), aminoacylase III activity. Positions of the size markers are
shown (arrows).
that the enzyme used in this study was not modified during the purification procedure and suggests that it should be structurally identical to native aminoacylase III.

**DISCUSSION**

The cloning and characterization of mouse aminoacylase III provide an opportunity to evaluate the characteristics of this enzyme, which preferentially catalyzes the N-acetylation of N-acyl aromatic amino acids. Although previous studies have characterized partially purified aminoacylase from rat liver (36) and rat kidney (13, 42), the potential role this enzyme plays in generating nephrotoxic products derived from mercapturates in proximal tubules will be more completely understood given that its primary and oligomeric structures are now known. In this regard, the cloned enzyme provides a potentially important therapeutic target to prevent the nephrotoxicity of various haloalkene- and hydroquinone-derived mercapturates.

Mouse aminoacylase III is a homotetramer of $\sim$140 kDa that consists of monomers of $\sim$35 kDa. Similar to this finding, partially purified rat liver aminoacylase had a molecular mass of 145 kDa and consisted of monomers of $\sim$35 kDa (36). In contrast, partially purified rat kidney aminoacylase had a molecular mass of 55 kDa (13, 42) and a monomer molecular mass of 33 kDa (42). These differences may reflect real differences in oligomeric structure between the enzymes from different species or the possible effects of isolation and purification conditions. In this regard, our findings are more compatible with the data previously obtained from rat liver (36). The oligomeric structure of murine aminoacylase III might be important for the regulation of its enzymatic activity. Nevertheless, a deviation from Michaelis kinetics was not detected in this study, which suggests that there is no interaction of monomers in mouse aminoacylase III that affects the binding of the substrates to the enzyme.

The mercapturates of nephrotoxic cysteine S-conjugates are not substrates for mitochondrial $\beta$-lyase because of a blocked amino group and therefore require deacetylation before $\beta$-lyase-mediated activation. It was previously hypothesized that the efficiency of the deacetylation reaction in different parts of the nephron could determine the nephrotoxic effects of a given mercapturate (10). Because it has been shown that $\beta$-lyase is nearly uniformly distributed in S1, S2, and S3 segments of proximal tubules (10, 23), this enzyme cannot be responsible for the varying degree of nephrotoxicity among these segments. Our immunolocalization data suggest that aminoacylase III is a potential candidate for mediation of the nephrotoxic effects of certain cysteine S-conjugates.

In S1 tubules, the enzyme was localized predominantly to the apical membrane, whereas in proximal straight tubules, the enzyme was localized in the cytoplasm. The predominant apical localization of aminoacylase III in the S1 segment is a potential clue that can explain the higher resistance of this nephron segment to certain mercapturic acids. Although speculative, the spatial localization of the enzyme may protect S1 mitochondria by 1) decreasing the likelihood that cysteine S-conjugates are bioactivated by mitochondrial cysteine S-conjugate $\beta$-lyase to nephrotoxic reactive thiols; and/or 2)...

### Table 1. Kinetic characteristics and pH optimum of mouse aminoacylase III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{max}$, $\mu$mol$\cdot$mg$^{-1}$, min$^{-1}$</th>
<th>$K_{m}$, mM</th>
<th>pH Optimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetyl-L-cysteine</td>
<td>11.7 ± 1.4</td>
<td>1.1 ± 0.11</td>
<td>7.5 ± 0.2</td>
</tr>
<tr>
<td>N-acetyl-L-tyrosine</td>
<td>5.9 ± 1.5</td>
<td>1.8 ± 0.23</td>
<td>7.6 ± 0.2</td>
</tr>
<tr>
<td>N-acetyl-L-phenylalanine</td>
<td>7.5 ± 1.1</td>
<td>1.4 ± 0.32</td>
<td>7.6 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE.
maintaining a favorable cell-to-lumen mercapturic acid concentra-
tion gradient, thereby enhancing the luminal influx of
mercapturic acids via MRP2. The latter mechanism requires
that the enzyme is localized to the luminal side of the apical
membrane as, for example, γ-glutamtransferase, which is
anchored to the brush border through a hydrophobic NH2-
terminal domain (37). Hydrophobicity analysis of aminocacyl-
ase III indicates that the enzyme lacks a potential transmem-
brane segment, which suggests that the mechanism for its
apical localization in S1 cells involves its interaction with one or
more apically localized proteins.

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