An aminopeptidase with a preference for N-terminal aspartyl and glutamyl residues but distinct from gluta-
myl aminopeptidase (EC 3.4.11.7) was purified to near homogeneity from rabbit brain cytosol. Its properties were similar to an enzyme described previously (Kelly, J. A., Neidle, E. L., and Neidle, A. (1983) J. Neurochem. 40, 1727–1734). Aspartyl aminopeptidase had barely detectable activity toward simple aminoacyl-naphthylamide substrates. Its activity was determined with the substrate Asp-Ala-Pro-naphthylamide in the presence of ex-
cess dipeptidyl-peptidase IV (EC 3.4.14.5). The native enzyme has a molecular mass of 440 kDa and migrates as a single band of 55 kDa after SDS-polyacrylamide gel electrophoresis. The sequences of three tryptic peptides were used to screen the GenBank™ data base of ex-
pressed sequence tags. Human and mouse clones de-
scribed as “similar to a yeast vacuolar aminopeptidase” and containing full-length cDNAs were identified and sequenced. The human cDNA was expressed in Esche-
richia coli. The amino acid sequence has significant ho-
moology to yeast aminopeptidase I, placing it as the first identified mammalian member of the M18 family of metal-
alloproteinases. Homologous sequences in Caenorhab-
ditis elegans and in prokaryotes revealed three con-
served histidines, three conserved glutamates and five con-
erved aspartates. Aspartyl aminopeptidase is found at relatively high levels in all mammalian tissues exam-
ined and is likely to play an important role in intracel-
ular protein and peptide metabolism.

Aminopeptidases catalyze the sequential removal of amino acids from the unblocked N termini of peptides and proteins. These enzymes are widely distributed in eukaryotes and pro-
karyotes (1, 2) as either integral membrane or cytosolic pro-
teins. Aminopeptidases are generally classified in terms of their substrate specificities, i.e. preference for a neutral, acidic, or basic amino acid in the P1 position. In the case of X-Pro aminopeptidase (aminopeptidase P; EC. 3.4.11.9), it is the amino acid in the P1’ position that governs specificity. Most aminopeptidases are metalloenzymes, although cysteine and serine aminopeptidases have been described (2). In addition to their role in general protein and peptide metabolism, amino-
peptidases have more specific functions. These include activa-
tion (3) and inactivation (4) of biologically active peptides, removal of the N-terminal methionine of newly synthesized proteins (5) and possibly in the trimming of antigens for pres-
entation by the major histocompatibility complex-I system (6).

The removal of N-terminal aspartyl and glutamyl residues from proteins and peptides in eukaryotes is catalyzed by glu-
tamyl aminopeptidase (aminopeptidase A; EC 3.4.11.7), an en-
zyme first described by Glenner and Folk in 1961 (7) (for a review, see Ref. 8). This membrane-bound ectoenzyme is a member of the metalloproteinase family M1 and contains the HEXXH + E zinc binding ligands (9). Glutamyl aminopepti-
dase, first cloned as the murine BP-1/6C3 antigen (10), is a

protein of 945 amino acids with a molecular mass of 107.8 kDa. The purified porcine, human, rat, and mouse enzymes are isolated as homodimers (8). A distinguishing feature of glu-
tamyl aminopeptidase is its stimulation by Ca²⁺ (11). The ability of glutamyl aminopeptidase to degrade angiotensins I and II is of considerable interest. The action of glutamyl ami-
nopeptidase on angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-
H) yields des-Asp angiotensin II, also known as angiotensin III. There is evidence that angiotensin III may mediate some of the effects of the renin-angiotensin system in the brain (12). A recent study utilizing 3-amino-4-thio-butyl sulfonate, a specific inhibitor of this enzyme, provided evidence for a predominant role of angiotensin III in the control of vasopressin release (13). The same inhibitor was used to establish a major role of glu-
tamyl aminopeptidase in the metabolism of cholecystokinin-8 (14).

The literature also contains two reports of a cytosolic acidic amino acid preferring aminopeptidase, which is distinct from glutamyl aminopeptidase. Cheung and Cushman (15) de-
scribed the partial purification of such an enzyme from the soluble fraction of a dog kidney extract. It is activated by preincubation with Mn²⁺ and has a preference for Asp-2-naph-
thyldiamine (Asp-NA) over Glu-NA. Kelly et al. (16) have more extensively characterized a high molecular weight (450,000) acidic amino acid specific aminopeptidase in mouse brain cy-
tosol. One of the features of the mouse brain enzyme is its inability to cleave simple NA substrates such as Asp-NA and Glu-NA. Due to its instability, this enzyme was not previously purified to homogeneity. In view of our interest in the role of angiotensin III in the brain renin-angiotensin system (17), we sought to further study the latter enzyme. A new assay was developed to measure its activity. We report on the purification, specificity, properties, subunit structure, molecular cloning, and expression of the enzyme we call aspartyl aminopeptidase.

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†† The abbreviations used are: NA, 2-naphthylamide; THF, tetrahy-
drofuran; DMF, dimethylformamide; Boc, N-tert-butoxycarbonyl; tert-butyl; Fmoc, 9-fluorenylmethoxycarbonyl; SM, sulfamethoxazole; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; DTT, dithiothreitol; Bicine, N,N-bis(2-hydroxyethyl)glycine.

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Aspartyl Aminopeptidase

Experimental Procedures

Materials

All peptide intermediates were obtained from Bachem Inc., (Philadelphi, PA). Frozen rabbit brains were obtained from Pel Freez (Rogers, AR). Freshly dissected rat tissues were obtained from rats sacrificed in the laboratory of Dr. Robert Blitzer of the Department of Pharmacology, Mount Sinai School of Medicine. The following compounds were obtained from Sigma: angiotensins II and III, 2-naphthylamine, aspartyl-2-naphthylamide, glutamyl-2-naphthylamide, phenylalanyl-naphthylamide, Lys-Ala-naphthylamide, leucine enkephalin, Pro-Leu-glycinamide, leucyl-p-nitroanilide, aspartyl-β-hydroxamate, sulfamethoxazole, pyrourcmycin, bacitracin, amastatin, and bestatin. The following compounds were obtained from Aldrich: 1-hydroxybenzotriazole, dicyclohexylcarbodiimide, N-methylmorpholine, isobutylcholorormate, Silica Gel Merck, grade 3835. Affi-Gel 22 was obtained from Sepracor Inc. (Marlborough, MA). Oligonucleotide primers were obtained from ABT Inc. (Corvalle, IA). Dipeptidyl-peptidase IV activity is defined as the amount of enzyme releasing 1 nmol of NA/b from Ala-Pro-NA. Glutamyl aminopeptidase was purified to apparent homogeneity from rat kidney as described (18). A unit of dipeptidyl-peptidase IV activity is defined as the amount of enzyme releasing 1 nmol of NA/b from Ala-Pro-NA. Glutamyl aminopeptidase was purified to apparent homogeneity from rat kidney by immunofaffinity chromatography (8).

Synthesis of Substrates

Ala-Pro-NA

Boc-Pro-NA—A mixture of 7 mmol (1 g) of NA and 7 mmol (1.5 g) of Boc-Pro in 10 ml of dry THF was cooled to −20 °C. Next 7 mmol each of isobutylcholorormate and N-methylmorpholine were added, and the reactants were stirred for 20 min at this temperature. The reaction mixture was allowed to come to room temperature and filtered. The precipitate was washed with THF, and the combined THF fractions were evaporated to dryness. The residue was dissolved in DMF, followed by 0.5 mmol each of 1-hydroxybenzotriazole and dicyclohexylcarbodiimide and 1-hydroxybenzotriazole were added. The solution was stirred at this temperature for 18 h and then filtered. The solvent was evaporated, and the residual was dissolved in chloroform and then washed with 10% citrate, and H2O. The organic layer was washed with 2 × 20 ml of saturated NaHCO3, 2 × 20 ml of H2O, and 20 ml of saturated NaCl. After drying over Na2SO4, the solvent was removed in vacuo. The residue was washed with 50 ml of ethyl ether and then treated with trifluoroacetic acid for 1 h. After removal of the trifluoroacetic acid under high vacuum, the product Glu-Ala-Pro-NA was obtained in 81% yield. M+ 1 = 441.

Fmoc-Asp-Ala-Pro-NA

Equimolar amounts of triethyamine and Fmoc-Asp(O-t-Bu)-OH (0.5 mmol) were added to a stirred solution of 0.5 mmol of compound 1 in 10 ml of DMF. The solution was cooled to 4 °C, and then 0.5 mmol each of dicyclohexylcarbodiimide and 1-hydroxybenzotriazole were added. The solution was stirred at this temperature for 18 h and then filtered. The solvent was evaporated, and the residue was dissolved in chloroform and then washed with 10% citrate, and H2O. After drying over Na2SO4, the solvent was removed in vacuo. The residue was washed with 50 ml of ethyl ether and then treated with trifluoroacetic acid for 1 h. After removal of the trifluoroacetic acid under high vacuum, the product Fmoc-Asp-Ala-Pro-NA was obtained in 83% yield. M+ 1 = 650.

Asp-Ala-Pro-NA

Triethyamine and Boc-Ala-N-hydroxysuccinimide ester (0.5 mmol each) were added to a stirred solution of 0.5 mmol of compound 1 in 10 ml of DMF. The mixture was allowed to stir overnight at room temperature. The solvent was then removed by evaporation, and the residue was dissolved in chloroform. The chloroform was sequentially washed with saturated NaHCO3, H2O, 10% citrate, and H2O. After drying over Na2SO4, the solvent was removed to yield 220 mg of Boc-Ala-Pro-NA. Treatment with trifluoroacetic acid for 30 min followed by evaporation and ether precipitation, yielded 186 mg of Fmoc-Asp-Ala-Pro-NA as white crystalline material (yield 29%; M+ 1 = 650).

Fmoc-Asp-Ala-Pro-NA

Equimolar amounts of triethyamine and Fmoc-Asp(O-t-Bu)-OH (0.5 mmol) were added to a stirred solution of 0.5 mmol of compound 1 in 10 ml of DMF. The solution was cooled to 4 °C, and then 0.5 mmol each of dicyclohexylcarbodiimide and 1-hydroxybenzotriazole were added. After drying over Na2SO4, the solvent was removed in vacuo. The residue was washed with 50 ml of ethyl ether and then treated with trifluoroacetic acid for 1 h. After removal of the trifluoroacetic acid under high vacuum, the product Glu-Ala-Pro-NA was obtained in 81% yield. M+ 1 = 441.

Peptide Synthesis

A series of tetrapeptides were synthesized on an Applied Biosystems model 430A peptide synthesizer by Fmoc chemistry. Peptide purity was evaluated by HPLC (see below).

Measurement of Enzymatic Activity

The activity of aspartyl aminopeptidase was measured by a coupled enzymatic assay similar to that described for the measurement of pyroglutamyl peptidase II (19). In this assay, the product of the action of aspartyl aminopeptidase is a substrate of dipeptidyl-peptidase IV (EC 3.4.14.5).

The reaction sequence is as follows,

Asp-Ala-Pro-CAspyl aminopeptidase → Asp + Ala-Pro-C

Dipeptidyl-peptidase IV → Ala-Pro + C

Reactions 1 and 2
Aspartyl Aminopeptidase

Enzyme Purification

Step 1: Preparation of Rabbit Brain Supernatant

Frozen rabbit brains (50 g) were partially defrosted and homogenized in a Waring blender with 4 volumes of 25 mM Bicine, pH 7.0, 5% glycerol (buffer A). The homogenate was centrifuged for 1 h at 100,000 × g, and the supernatant was retained. The pellet was washed with an equal volume of buffer A and centrifuged for 1 h at 100,000 × g, and the supernatant fractions were combined.

Step 2: Affi-Gel Blue Chromatography

The supernatant was passed over a 2.5 × 20-cm column of Affi-Gel blue, 100–200 mesh, equilibrated with buffer A. The enzymatically active fractions of the effluent were combined.

Step 3: Q-Sepharose Chromatography

The enzymatically active fractions were applied to a 5 × 15-cm Q-Sepharose column, equilibrated with buffer A, and the column was then washed with 1500 ml of buffer A. The enzyme was eluted with a 2-liter 0–0.4 M NaCl gradient in buffer A. The active fractions were combined and concentrated in an Amicon ultrafiltration cell fitted with a PM-10 membrane.

Step 4: Aca-22 Gel Filtration

The concentrated enzyme was applied to a 2.5 × 90-cm Aca-22 column equilibrated with 50 mM Bicine, pH 7.0, 10% glycerol, 0.1 mM NaCl. The active fractions were combined, and NaCl was added to a final concentration of 0.3 mM.

Step 5: Phenyl-Sepharose Chromatography

The active fractions were chromatographed on a 1 × 7-cm phenyl-Sepharose column equilibrated with 50 mM Bicine, pH 7.0, 10% glycerol, 0.3 mM NaCl (buffer B). The column was washed with 30 ml of buffer B. The enzyme was eluted with 30 ml of 50 mM Bicine, pH 7.0, 60% glycerol. The active fractions were dialyzed against 10 mM sodium phosphate buffer, pH 7.0, 10% glycerol (buffer C).

Step 6: Hydroxylapatite Chromatography

The dialyzed enzyme was applied to a 2.5 × 1-cm column of hydroxyapatite equilibrated with buffer C, and the column was washed with this buffer. The effluent was collected, and fractions containing active enzyme were concentrated and then stored at −80 °C.

Sequence Determination of Tryptic Peptides

Microsequencing was conducted at the W. M. Keck Foundation Biotechnology Resource Laboratory of Yale University. Gel slices containing protein were diced with a razor blade and the eluate was subjected to microbore HPLC. Matrix-assisted laser desorption/ionization mass spectrometry was used to evaluate peak purity and identify candidate peaks suitable for sequencing. Two sequences were obtained, and a third sequence of a tryptic peptide was obtained from the Baker Medical Research Institute (Prahran, Australia).

Nucleotide Sequencing

Nucleotide sequencing of ATCC clone 355977 was performed by the Biotechnology Center, Utah State University (Logan, UT) using sequence-specific oligonucleotide primers synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Construction of the pSE-6HDAP Expression Plasmid

The cDNA coding for aspartyl aminopeptidase was excised from ATCC clone 355977 (lafmid BA plasmid) by first digesting with NotI and then partially digesting with BstXII to release a 1.55-kilobase pair fragment containing the entire coding region minus 174 bp from the 5′-end. An adaptor was synthesized containing a 5′-overhang complementary to an NcoI digestion site on the 5′-end and a 3′-overhang complementary to the BstXI-generated overhang on the 3′-end. This adaptor contained the ATG start codon followed by six histidine codons, a glycine codon, and then the codon for asparagine normally present after the putative start codon in the aspartyl aminopeptidase sequence. The sequences of the oligonucleotides annealed to make the adaptor were 5′-CATGCACCATACCAACACTACGCCAGGAC-3′ and 5′-GCGACTAC-

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\[ \text{Construction of the pSE-6HDAP Expression Plasmid} \]

where C represents a chromogen. Initially, we used 2-naphthylamine as chromogen, and more recently we have used the less toxic and more soluble sulfamethoxazole. The released chromogen is measured by a colorimetric procedure following its diazotization (20).

The molecular mass of the native enzyme was determined by FPLC 

\[ \text{Determination of Molecular Mass of the Native Enzyme} \]

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\[ \text{Construction of the pSE-6HDAP Expression Plasmid} \]
confirmed by restriction digestion. The cells containing the plasmid were grown in the presence of 2 mM isopropyl-1-thio-

galactopyranoside, and aspartyl aminopeptidase activity was determined.

Northern Blot

Total RNA was prepared essentially by the method of Chomczynski and Sacchi (24) from freshly frozen rat tissues. Formaldehyde gel

electrophoresis and transfer of 20 μg of RNA/lane were performed using the

methods described in Sambrook et al. (25). The RNA was transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech) and

hybridized with 40 mCi of a mouse aspartyl aminopeptidase cDNA probe from ATCC clone 355377, which had been labeled to a specific activity

of approximately 0.1 μCi/μg with [α-32P]dCTP using the NEBlot kit from New England Biolabs. Prehybridization and hybridization at 42 °C were carried out in 20 ml of 6x SSC, 0.1% SDS at 42 and 55 °C followed by a high stringency wash in 0.1x SSC, 0.01% SDS at 55 °C An RNA ladder from Life Technologies, Inc. was also electrophoresed for calculation of molecular size.

RESULTS

The cytosol of a rabbit brain homogenate contained an enzyme that cleaved the substrate Asp-Ala-Pro-NA and that, in the presence of excess dipeptidyl-peptidase IV, liberated free naphthylamine. Enzymatic activity was optimal in the neutral pH range (Fig. 1). This enzyme having the properties of an aspartyl aminopeptidase (see below) was purified 722-fold by conventional chromatographic techniques from a rabbit brain supernatant, with an overall yield of 3.8% (Table I). Starting with 50 g of rabbit brain, 112 μg of enzyme protein was obtained. Examination of the enzyme by SDS-PAGE revealed a highly purified preparation containing a major protein band and trace amounts of higher molecular weight bands (Fig. 2).

The molecular mass of the native enzyme was determined by gel filtration chromatography on a calibrated FPLC Superose 6 column. The enzyme eluted identically with ferritin, establishing a molecular mass of 440 kDa. This value is virtually identical to the estimate of 450 kDa reported by Kelly et al. (16). SDS-PAGE gave a monomer molecular mass of 55 kDa.

The specificity of the enzyme was explored with a series of peptides. Asp-Ala-Pro-SM and Asp-Ala-Pro-NA used for assay and purification were cleaved at the Asp-Ala bond, since release of the chromogen was dependent upon the presence of dipeptidyl-peptidase IV. The $k_{cat}/K_m$ ratio for Asp-Ala-Pro-NA exceeded that of the corresponding glutamyl peptide. The difference was primarily reflected in $K_m$ values with the glutamyl peptide binding very poorly to the enzyme. The enzyme required the presence of a free α-amino group. When the N terminus of the substrate Asp-Ala-Pro-NA was blocked by an Fmoc group, there was no cleavage. The enzyme also required the presence of an acidic amino acid at the N terminus for optimal activity. There was no measurable cleavage (<1% of Asp-Ala-Pro-NA) for Ala-Ala-Pro-NA, Lys-Ala-NA, Pro-Leu-Gly-NH₂, leucine enkephalin (Tyr-Gly-Gly-Phe-Leu), Ile-His-Pro-Phe or Ser-Ala-Ala-Leu (also see below). An asparaginyl peptide (Asn-Ala-Ala-Leu) was cleaved at 5% of the rate of Asp-Ala-Pro-NA. Simple naphthylamides or p-nitroanilides such as Asp-NA, Glu-NA, Phe-NA, and Leu-pNA were also negligibly cleaved.

The subsite specificity of aspartyl aminopeptidase was explored with a series of aspartyl tetrapeptides and compared with the subsite specificity of glutamyl aminopeptidase (Table II). HPLC was used for analysis of the products of reaction and quantitation of the rate of substrate disappearance. The $K_i$ of the peptides competing with the chromogenic substrate Asp-Ala-Pro-NA (for aspartyl aminopeptidase or Glu-NA (for glutamyl aminopeptidase) was determined by the method of Dixon (23). These $K_i$ values were used as a measure of $K_m$. The subsite specificities of both enzymes are similar. Neutral or hydrophobic amino acids in the P1′ position are preferred. For this series of tetrapeptides, a positively charged amino acid in the P1′ position has a particularly adverse effect on the specificity constant (however, see below). Moreover, substitution in the P2′ position can also strongly affect the $k_{cat}/K_m$ ratio. For
Aspartyl Aminopeptidase

A comparison of specificity constants of aspartyl peptides for aspartyl aminopeptidase and glutamyl aminopeptidase

| Peptide (P1-P1’-P2’-P3’) | Aspartyl aminopeptidase | Glutamyl aminopeptidase |
|---------------------------|-------------------------|-------------------------|
|                           | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|                           | $s^{-1}$ | $\mu A$ | $s^{-1}/\mu A$ | $s^{-1}$ | $\mu A$ | $s^{-1}/\mu A$ |
| Asp-Ala-Ala-Leu           | 5.3      | 0.6   | 8.8 x 10^2   | 6.9      | 0.09  | 7.7 x 10^4   |
| Asp-Phe-Ala-Leu           | 9.9      | 1.3   | 7.6 x 10^3   | 11.8     | 0.18  | 7.4 x 10^4   |
| Asp-Lys-Ala-Leu           | 2.8      | 2.2   | 1.3 x 10^3   | 2.3      | 0.3   | 7.7 x 10^4   |
| Asp-Asp-Ala-Leu           | 9.8      | 2.8   | 3.5 x 10^3   | 5.6      | 0.18  | 3.1 x 10^4   |
| Asp-Ala-Phe-Leu           | 17.2     | 0.4   | 4.3 x 10^4   | 6.3      | 0.61  | 1.0 x 10^4   |
| Asp-Ala-Lys-Leu           | 5.0      | 1.5   | 3.3 x 10^3   | 3.6      | 0.90  | 4.0 x 10^4   |
| Asp-Ala-Asp-Leu           | 2.3      | 0.05  | 4.6 x 10^4   | 6.1      | 0.17  | 3.6 x 10^4   |
| Angiotensin II            | 6.8      | 0.25  | 2.7 x 10^4   | 1.4      | 0.035 | 4.0 x 10^4   |
| (Asp-Arg-Tyr-Ile-His-Pro-Phe) |         |       |              |          |       |             |
| Asp-Ala-Pro-NA            | 12.4     | 2.0   | 6.2 x 10^3   |          |       |              |
| Glu-Ala-Pro-NA            | 19.8     | 8.4   | 2.4 x 10^3   |          |       |              |
| Asp-Ala-Pro-SM            | 4.4      | 1.0   | 4.4 x 10^3   |          |       |              |

The $K_v$ for competition of the peptides with the substrate Asp-Ala-Pro-NA for aspartyl aminopeptidase and with the substrate Glu-NA for glutamyl aminopeptidase was determined by the method of Dixon (23) and equated with $K_m$. Each determination was carried out at two substrate concentrations and six concentrations of competing peptide. Each experimental point was run in triplicate. The average correlation coefficient for the generated lines was 0.9. The errors of the $K_v$ values were calculated by a determination of the combined errors of the slopes and y-intercepts of the lines fitted by least squares regression. The errors ranged from ±7.5% for the substrate DFAL, acted upon by glutamyl aminopeptidase, to ±31.5% for the substrate DDAL, acted upon by aspartyl aminopeptidase. HPLC was used to quantitate the time-dependent degradation of the peptides, and degradation was measured at five time points. The specificity constants reported in this table represent single determinations.

For glutamyl aminopeptidase, the incubation mixtures contained 1000 nmol of peptide, 0.5 µg of enzyme, 2 mM Ca²⁺ and 0.05 mM Tris-HCl buffer, pH 7.5, in a total volume of 0.5 ml. For aspartyl aminopeptidase, incubation mixtures contained 1000 nmol of peptide, 0.7 µg of enzyme, and 0.05 mM Tris-HCl, pH 7.5 in a total volume of 0.5 ml. The incubation mixtures with angiotensin II contained the following: for aspartyl aminopeptidase, 230 nmol of angiotensin II, 0.25 µg of enzyme, and Tris-HCl buffer (0.05 M; pH 7.5) in a total volume of 125 µl; for glutamyl aminopeptidase, 90 nmol of angiotensin II, 1 µg of enzyme, 4 mM Ca²⁺, and Tris-HCl buffer (0.05 M; pH 8.3) in a total volume of 125 µl. The following peptides were not detectably degraded (<1% of the rate of Asp-Ala-Pro-NA): Ala-Ala-Pro-NA, Lys-Ala-Pro-NA, angiotensin III (Arg-Val-Tyr-Ile-His-Pro-Phe), leucine enkephalin (Tyr-Gly-Gly-Phe-Leu), Pro-Leu-Gly-NH₂, Ile-His-Pro-Phe, and Ser-Ala-Ala-Leu. Asn-Ala-Ala-Leu was degraded at a rate of 5% of Asp-Ala-Pro-NA.

For example, with aspartyl aminopeptidase, the $k_{cat}/K_m$ ratio for Asp-Ala-Phe-Leu is 13-fold greater than the $k_{cat}/K_m$ ratio for Asp-Ala-Lys-Leu. In general, the specificity constants for substrates acted upon by glutamyl aminopeptidase were greater than the specificity constants of the same substrates cleaved by aspartyl aminopeptidase.

The effect of glutamyl and aspartyl aminopeptidases on the degradation of angiotensin II was also compared. Incubation of angiotensin II with either enzyme led to the production of angiotensin III (des-Asp-angiotensin II) as the only product. Prolonged incubation with aspartyl aminopeptidase, resulting in a total degradation of angiotensin II, did not lead to the formation of products other than angiotensin III (Fig. 3). These studies therefore directly demonstrate the specificity of aspartyl aminopeptidase for N-terminal acidic amino acids. Both the $k_{cat}$ and $K_m$ of angiotensin II measured with aspartyl aminopeptidase exceeded the corresponding values measured with glutamyl aminopeptidase; however, the $k_{cat}/K_m$ ratios were similar (Table II). It should be noted that angiotensin II contains a positively charged amino acid in the P1 position yet has a 20-fold greater specificity constant compared with the tetrapeptide Asp-Lys-Ala-Leu (Table II). This implies that the enzyme may prefer octapeptides to tetrapeptides. However, to substantiate this conclusion, the effect of peptide length on the specificity constant would have to be systematically explored, since the substrates in the P1’-P3’ positions of angiotensin II differ from the corresponding substituents in the tetrapeptide.

The effect of a series of proteinase inhibitors or potential activators on enzyme activity was studied (Table III). In these experiments, each compound was tested without preincubation and after 15 min preincubation. The enzyme was sensitive to longer preincubation periods. A 30-min preincubation led to loss of about one-third of the activity. Unlike glutamyl aminopeptidase, aspartyl aminopeptidase was not stimulated by Ca²⁺, and, unlike the activity described by Cheung and Cushman (15), aspartyl aminopeptidase was not stimulated by Mn²⁺. Zn²⁺ at a concentration of 0.4 mM inhibited activity by 31.5% for the substrate DFAL, acted upon by aspartyl aminopeptidase. HPLC was used to quantitate the time-dependent degradation of the peptides.
Enzyme activity was measured with the substrate Asp-Ala-Pro-NA as described under “Experimental Procedures.” Inhibitors or activators added without preincubation and after a 15-min preincubation. Results are expressed as mean ± S.E. for three determinations each. The following compounds had no activity either with or without preincubation: bestatin (0.1 mM); amastatin (10 μM); puromycin (0.4 mM); NaCl (10 mM), and aspartyl-β-naphthylamide (0.4 mM).

| Compound               | Conc. | Activity (percentage of control) | 0-min preincubation | 15-min preincubation |
|------------------------|-------|---------------------------------|---------------------|----------------------|
| Dithiothreitol         | 1.0   | %                               | 12 ± 0.3            | 11 ± 0.3             |
|                        | 0.1   |                                 | 25 ± 4.6            | 18 ± 3.0             |
|                        | 0.01  |                                 | 75 ± 2.5            | 79 ± 7.5             |
| Zn²⁺                   | 0.4   |                                 | 47 ± 5.9            | 0                    |
| Co²⁺                   | 0.4   |                                 | 105 ± 6.7           | 78 ± 2.2             |
| Ca²⁺                   | 4.0   |                                 | 105 ± 6.7           | 114 ± 16             |
| Mn²⁺                   | 4.0   |                                 | 84 ± 4.7            | 111 ± 11             |
| Bacitracin             | 0.02  |                                 | 145 ± 1.8           | 205 ± 3.8            |
| Aspartyl-β-hydroxamate | 0.4   |                                 | 41 ± 2.6            | 48 ± 8.7             |
| p-Chloromercuribenzoate| 0.4   |                                 | 92 ± 2.9            | 87 ± 3               |
| EDTA                   | 5.0   |                                 | 102 ± 6.3           | 107 ± 5              |
|                        | 20.0  |                                 | 104 ± 4.8           | 99 ± 1               |
| o-Phenanthroline       | 2.4   |                                 | 49 ± 1.9            | 1 ± 0.3              |

FIG. 4. Activation of aspartyl aminopeptidase by bacitracin.

The enzyme was incubated with varying amounts of Asp-Ala-Pro-NA in the absence (●) and in the presence (○) of bacitracin (100 μM). Enzymatic activity was determined as described under “Experimental Procedures.” Reaction velocity is expressed in absorbance units at 580 nm.

TABLE IV

| Tissue  | Activity (μmol/mg protein/h) |
|---------|-----------------------------|
| Brain   | 0.99 ± 0.21                 |
| Heart   | 0.75 ± 0.09                 |
| Intestine | 1.39 ± 0.34               |
| Kidney  | 1.42 ± 0.19                 |
| Liver   | 0.96 ± 0.12                 |
| Lung    | 0.98 ± 0.21                 |
| Spleen  | 0.81 ± 0.12                 |
| Testes  | 3.48 ± 0.39                 |

Activity was determined in the presence of puromycin and Zn²⁺ as described under “Experimental Procedures.” Results are presented as mean ± S.E. for three samples. Each sample was determined in triplicate.

Aspartyl Aminopeptidase

The activity of the enzyme was determined in the cytosolic fraction of rat tissues. These assays were conducted in the presence of 0.4 mM Zn²⁺ to inhibit any contaminating glutamyl aminopeptidase. Although this concentration of zinc inhibits purified aspartyl aminopeptidase by about 50% (Table III), it approximately 50% without preincubation but totally inhibited after 15 min of preincubation. An interesting feature of aspartyl aminopeptidase is its sensitivity to dithiothreitol (DTT). A concentration of 1 mM DTT inhibited enzymatic activity by 88%. We considered the possibility that disulfide bonds were necessary for the quaternary structure of the enzyme and that DTT may inhibit by dissociating the oligomer. Accordingly, we examined the electrophoretic profile of aspartyl aminopeptidase in buffers containing DTT. No evidence of dissociation was observed (data not shown). The inhibition by DTT may be due to metal ion chelation. Preincubation with o-phenanthroline led to virtually a total loss of activity; however, aspartyl aminopeptidase was relatively insensitive to EDTA. Prolonged dialysis against EDTA buffers was necessary to observe inhibition. After 120 h of dialysis against 10 mM EDTA, an approximate 67% inhibition was observed. Activity could be fully restored by the addition of Zn²⁺. In these experiments, the concentration of EDTA in the incubation mixtures was 0.8 mM. Activity was fully restored by Zn²⁺ at final concentrations of 1.2, 1.6, and 2.4 mM. The aminopeptidase inhibitors amastatin and bestatin were without effect. At the concentration of amastatin used, membrane alanyl aminopeptidase was totally inhibited, and glutamyl aminopeptidase was 50% inhibited. Bacitracin unexpectedly stimulated enzymatic activity (Table III; Fig. 4). A Lineweaver-Burk analysis of the experiment shown in Fig. 4 demonstrated that 200 μM bacitracin lowered the Kₘ from 2.2 to 1.6 mM while moderately increasing Vₘₐₓ by 15%. Activation was moderately enhanced by preincubation. The enzyme was sensitive to p-chloromercuribenzoate, a feature commonly observed with cystolic proteinases. We also measured the effect of chloride ions, since chloride was reported to stimulate yeast aminopeptidase I (see below). It should be noted that all metal ions reported in Table III were tested as chloride salts. No activation by 40 mM calcium chloride was observed, nor was there activation by 10 mM NaCl.

The activity of the enzyme was determined in the cytosolic fraction of rat tissues. These assays were conducted in the presence of 0.4 mM Zn²⁺ to inhibit any contaminating glutamyl aminopeptidase. Although this concentration of zinc inhibits purified aspartyl aminopeptidase by about 50% (Table III), it does not inhibit the enzyme in crude tissue extracts. Aspartyl aminopeptidase was present in all tissues studied, and its distribution was fairly uniform. The highest activity was found in testes. There was an approximately 5-fold differential in specific activity of highest to lowest values (Table IV).

Cloning.—The electrophoretically homogeneous protein was subjected to trypsinization and the fragments separated by microbore HPLC. Two sequences were obtained from the Yale University facility. These were GFFELFPSLSR and LLQAGFHELK. Two sequences were obtained from the Yale University facility. These were GFFELFPSLSR and LLQAGFHELK. Two sequences were obtained from the Yale University facility. These were GFFELFPSLSR and LLQAGFHELK. Two sequences were obtained from the Yale University facility. These were GFFELFPSLSR and LLQAGFHELK. Two sequences were obtained from the Yale University facility. These were GFFELFPSLSLR and LLQAGFHELK. One sequence, LVQVEFPILR, was obtained by the Baker Medical Research Institute. No matches were found in the Protein Data Bank for any of these sequences. Some homology was noted for the peptide LVQVEFPILR and a Mycobacterium leprae sequence described as “vacuolar aminopeptidase I precursor” (LVRIIDDPILR). The same data base when screened with the sequence LLQAGFHELK revealed a sequence in the Caenorhabditis elegans genome (accession number U13070) described as similar to yeast vacuolar aminopeptidase (accession number M25548). The C. elegans sequence was used to search the expressed sequence tag data base best of GenBank™, and this search revealed mouse and human expressed sequence tags described as being similar to yeast vacuolar aminopeptidase. It was possible to locate sequences homologous to all three tryptic peptides of the rabbit brain.
enzyme in the mouse and human expressed sequence tags. Clones containing the 5'-end of the sequence were identified. The human clone (ATCC number 355377) contained an insert size of 1.791 kilobase pairs, sufficiently large to encode the entire reading frame of aspartyl aminopeptidase. This clone was sequenced, and its sequence is shown in Fig. 5. The molecular weight of the protein was calculated as 52,065, in good agreement with the value of 55,000 of purified aspartyl aminopeptidase determined by SDS-PAGE. When the full-length human sequence was entered into the blastp nr data bank, sequences were found with high homology from yeast, M. leprae (accession number U15182), and from a Lyme disease spirochete described as an aminopeptidase I-like protein (accession number S44086). One of the yeast sequences was that of aminopeptidase I (EC 3.4.11.22), and the other sequence was described as "hypothetical 54.2-kDa protein in CDC12-ORC6 intergenic region" (accession number P38821). An alignment of the yeast, human, mouse, C. elegans, M. leprae, and Lyme disease spirochete sequences is shown in Fig. 6.

An examination of these sequences revealed three conserved histidines corresponding to amino acids 94, 170, and 440 of the human sequence. There are also three conserved glutamates corresponding to amino acids 52, 301, and 302 of the human sequence and five conserved aspartates corresponding to amino acids 96, 264, 299, 356, and 431 of the human sequence (Fig. 6).

The cDNA for the cloned human enzyme was expressed as a His6 fusion protein in E. coli (Table V). Hydrolysis of the substrate Asp-Ala-Pro-SM by the nontransfected bacteria was very low, and activity was diminished when dipeptidyl-peptidase IV was omitted from the incubation mixture. The specific activity of Asp-Ala-Pro-SM in cells transfected with the cDNA of the cloned human enzyme varied from 5.5 to 47.6 units/mg in different groups. The mean activity was 90-fold greater than nontransfected cells and exceeded the activity found in crude extracts of testes (3.48 units/mg; Table IV). The Km for the expressed enzyme measured with the substrate Asp-Ala-Pro-SM (2.3 mM) was similar to that of the purified rabbit brain enzyme (2.0 mM). Hydrolysis was dependent on dipeptidyl-peptidase IV, demonstrating that the expressed enzyme cleaved the Asp–Ala bond of the substrate. The activity was moderately inhibited by 0.4 mM Zn2+ and unaffected by 0.4 mM puromycin. When the β-carboxyl-blocked compound Asp(O-t-Bu)-Ala-Pro-NA was substituted as substrate, activity was reduced by 98%. Thus, the expressed enzyme has all the characteristics of aspartyl aminopeptidase.

Northern blot analysis indicated a single mRNA species of 1.9 kilobase pairs in all tissues tested (Fig. 7). The largest amount of RNA was in testes; intermediate amounts were in kidney and lung; and lesser but significant amounts were in spleen, liver, and brain. These results were roughly comparable with enzyme activity, with the exception of brain, which showed similar enzyme activity to lung but considerably less RNA. Additional experiments (not shown) also demonstrated that the same message was present in intestine and heart.

**DISCUSSION**

The presence of an acidic amino acid-prefering aminopeptidase in mouse brain cytosol was documented more than a decade ago by Kelly et al. (16). This enzyme was described as the major aminopeptidase in brain homogenates that degrades N-terminal acidic amino acid-containing peptides. These investigators noted the instability of this enzyme. We have found that the activity could be stabilized by 5–10% glycerol-containing buffers. It was therefore possible to purify the enzyme to near homogeneity by conventional chromatographic procedures. The final preparation is stable when stored at –20 °C in the presence of 10% glycerol. The inability of this enzyme to cleave simple substrates such as Asp-NA and Glu-NA likely explains why this enzyme has been largely overlooked.

Aminopeptidases are routinely assayed by the use of simple aminoacyl-arylamide substrates. For example, Iribar et al. (27) report on the presence of a cytosolic aspartyl aminopeptidase in rat frontal cortex, which was measured with Asp-NA. The activity reported in pmol/min/g of tissue is 3 orders of magni-
tude lower than activity measured with Asp-Ala-Pro-SM as described under “Experimental Procedures.” Values are presented as mean ± S.E. Number of samples is shown in parentheses.

| Treatment | Activity (µmol/mg protein/h) |
|-----------|-----------------------------|
| Nontransfected cells | 0.22 ± 0.007 (5) |
| Nontransfected cells minus dipeptidyl-peptidase IV | 0.09 ± 0.008 (5) |
| Transfected cells | 19.4 ± 5.2 (8) |
| Transfected cells minus dipeptidyl-peptidase IV | 0.13 ± 0.02 (8) |
| Transfected cells + 0.5 mM Zn²⁺ | 16.3 ± 7.0 (8) |
| Transfected cells assayed with Asp(O-tBu)-Ala-Pro-NA | 0.47 ± 0.07 (8) |

Aspartyl aminopeptidase was expressed in E. coli as a His₆ fusion protein. The properties of the expressed enzyme are similar to the enzyme isolated from brain (Table V), indicating that the cDNA does indeed encode an aspartyl aminopeptidase.
and that the His$_6$ linker does not affect the catalytic properties of the enzyme. The level of expression is very high, and the presence of the His$_6$ group makes it possible to rapidly purify the enzyme on a nickel-nitrilotriacetic acid column (Qiagen). We have found that an approximately 90% pure preparation can be obtained by adsorption of the His$_6$ protein in a crude cell lysate on the nickel-nitrilotriacetic acid column followed by a 20 mM imidazole buffer wash and elution with a 200 mM imidazole buffer (not shown). Therefore, it is now possible to obtain large amounts of recombinant enzyme for further studies including crystallization.

The Northern blot analysis indicates a wide tissue distribution for aspartyl aminopeptidase, with the same 1.5-kilobase pair RNA species expressed in all tissues tested but with some tissue variation in the amount in relation to enzyme activity (i.e. brain versus lung). This may be due to the presence of differing amounts of endogenous inhibitors or activators or to variations between experimental animals. In any case, the ubiquitous nature of aspartyl aminopeptidase in various tissues and in evolution indicates that it must serve an important metabolic function or functions.

The sequences of the human and mouse aspartyl aminopeptidase cDNAs have significant homology to yeast aminopeptidase, with the same 1.9-kilobase pair RNA species expressed in all tissues tested but with some tissue variation in the amount in relation to enzyme activity (i.e. brain versus lung). This is probably due to a requirement for an acidic side chain in the P1 position for effective binding to aspartyl aminopeptidase.

The yeast genome does contain a sequence encoding a hypothetical 54.2-kDa protein (accession number P38821). This protein is more closely related to human aspartyl aminopeptidase (42.7% identity, Gene Stream align) than it is to yeast aminopeptidase I (30.2% identity) (Fig. 8). This hypothetical protein likely encodes the yet to be characterized yeast aspartyl aminopeptidase.

We have found that expressed sequence tags encoding aspartyl aminopeptidase have been mapped to chromosome 2q by both the Stanford Human Genome Center (markers SHGC 114 and SHGC 32022) and The Whitehead Institute Center for Genome Research (marker SGC-32022). The protein has been designated as "highly similar to hypothetical 54.2 kDa protein in CDC12-ORC6 intergenic region (Saccharomyces cerevisiae)" (55% similarity). The Stanford mapping places the gene at 209–220 cM, whereas the Whitehead mapping places the gene at 226–228 cM. The reason for the discrepancy is not clear.

On the basis of its homology to yeast aminopeptidase I, aspartyl aminopeptidase can be tentatively designated as a zinc metalloproteinase. The putative catalytic zinc ion of aspartyl aminopeptidase is very tightly bound. This is shown by the resistance of the enzyme to inactivation by EDTA. Moreover, the enzyme is purified from Bicine-buffered solutions, and Bicine itself is a strong metal ion chelator (28). Prolonged dialysis against EDTA is required to demonstrate significant inhibition, and enzymatic activity can be fully restored by the addition of Zn$^{2+}$. Preincubation with o-phenanthroline, however, does result in strong inhibition.

Although the subunit sizes of yeast aminopeptidase I and aspartyl aminopeptidase are similar, the native molecular mass of the yeast enzyme (640 kDa) is considerably higher than that of aspartyl aminopeptidase (440 kDa). Yeast aminopeptidase I has been described as a dodecameric protein (28). Aspartyl aminopeptidase may be composed of eight identical subunits, similar to ovine brain glutamine synthetase (31);
however, further studies will be required to determine its quaternary structure. High molecular masses and oligomeric structures are common features of cytosolic aminopeptidases. Aminopeptidase H with a molecular mass of 400 kDa and a subunit molecular mass of 52 kDa appears to have a similar structure to aspartyl aminopeptidase (32). Bleomycin hydrolase (a cysteine proteinase (33)) and leucine aminopeptidase (a metalloproteinase (34)) have homohexameric structures. The active sites of bleomycin hydrolase have been localized to the central channel of the hexamer (35). It is not known if a similar localization will be found for the active sites of aspartyl aminopeptidase.

Mammalian aspartyl aminopeptidase has high homology to a sequence in the C. elegans genome and also to sequences found in M. leprae and the Lyme disease spirochete. The latter three sequences are only putative aminopeptidases, since their enzymatic activities have not been determined. Although these proteins are likely to be members of the M18 metalloproteinase family, it is not known if their substrate specificities resemble aspartyl aminopeptidase or yeast aminopeptidase I (a leucyl aminopeptidase). The nature of the active site of yeast aminopeptidase I is unknown, since this zinc metalloproteinase lacks the classical zinc signature HEXXH (29). Amino acids serving as ligands for zinc are histidine, cysteine, glutamate, and aspartate (36). An alignment of the seven related sequences reveals three conserved histidyl residues, three conserved glutamyl residues, and five conserved aspartyl residues (Fig. 6). Site-directed mutagenesis should reveal whether any of these conserved residues are essential for enzymatic activity. Such studies should provide valuable information on the mechanism of action of a new class of metalloproteinase and lead to the rational design of specific inhibitors.

An examination of the sequence by PROSITE reveals putative phosphorylation sites for cAMP-dependent protein kinase, casein kinase II and protein kinase C. It is not known at this time if aspartyl aminopeptidase is phosphorylated at any of these sites and if so whether phosphorylation serves a regulatory function. The sequence contains no transmembrane regions and appears to represent a cytosolic protein.

The question of the physiological significance of aspartyl aminopeptidase awaits further study; however, several possibilities can be considered. The relatively high concentration of this enzyme in the cytosol of various tissues (>0.1% of soluble protein) and its fairly uniform tissue distribution suggest a role in general intracellular protein and peptide metabolism (37). An interesting exogenous substrate is aspartame (aspartyl-phenylalanine methyl ester) (16). A more specialized role in specific tissues may be its metabolism of biologically active peptides such as angiotensin II, cholecystokinin-8, and neuropeptide K. The activity of cytosolic aspartyl aminopeptidase was reported as decreased in the frontal cortex of aged rats (27). In this respect, it is of interest to note that β-amyloid peptide contains an N-terminal aspartyl residue, and a reduction in the removal of this residue has been proposed to facilitate β-amyloid deposition in Alzheimer’s disease (38). It has recently been proposed that aminopeptidases mediate the N-terminal trimming of peptides presented by the major histocompatibility complex-1 system (39). Thus presentation of N-terminal extensions of the immunodominant chicken ovalbumin peptide SIINFEKL is not blocked by proteasome inhibitors. The amino acid immediately preceding SIINFEFL is glutamate, and ESINFEKL should be an excellent substrate for aspartyl aminopeptidase but a very poor substrate for cytosolic leucine aminopeptidase. Identification of endogenous substrates of aspartyl aminopeptidase will be of obvious importance.

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