Molecular Cloning and Expression of Rat Liver Aminopeptidase B*

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We isolated, by immunological screening of a Uni-ZAP XR cDNA library constructed from rat liver mRNAs, a cDNA clone with 2212 base pairs encoding aminopeptidase B (EC 3.4.11.6). The open reading frame encodes a 649-amino acid protein with a theoretical molecular mass of 72,545 Da and bears the consensus sequence of the zinc metalloexopeptidases, indicating that the enzyme belongs to this family, which includes aminopeptidase A, aminopeptidase N, and leukotriene-A₄ hydro-lase. Escherichia coli SOLR cells infected with the pBluescript phagemid excised from the Uni-ZAP XR vector containing the aminopeptidase B cDNA had a high l-arginyl-b-naphthylamidase activity. The recombinant protein was purified to homogeneity from the recombinant E. coli extracts. The enzyme had Cl⁻-dependent aminopeptidase activity specifically restricted to the Arg and Lys derivatives and contained 1 mol of zinc per mol of the enzyme.

Aminopeptidases (Ap₁) play critical roles in processes such as protein maturation, protein digestion in its terminal stage, regulation of hormone levels, selective or homeostatic protein turnover, and plasmid stabilization (1).

These enzymes generally have broad substrate specificity and occur in several forms. Of such enzymes, Ap-B (EC 3.4.11.6), which was discovered in 1964 from a rat duodenal supernatant fraction (2), catalyzes specifically the removal of unsubstituted, N-terminal Arg and Lys residues from peptides and b-naphthylamide (NA) or other synthetic derivatives. The rate of arginine release is typically about twice that of lysine (3). Although there are numerous reports on the enzyme, conclusive evidence on the characterization of the enzyme is restricted to its Cl⁻-dependent activation (4) and sensitivity to inhibition by bestatin (5) and Arphamenine B (6). Since earlier reports based on inhibition and restoration experiments utilizing metal chelators and divalent metal ions (4, 7, 8), Ap-B has been regarded as a metalloenzyme; but there is some controversy on this point. Söderling and Mäkinen (9) used atomic absorption spectrophotometry to measure the zinc content of this enzyme, but they detected no zinc. On the other hand, Ocain and Rich (10) reported that this enzyme was inhibited by l-lysine thiol, a compound that would be expected to inhibit the enzyme by interaction with a catalytic zinc ion in the active site. In the current study, we cloned Ap-B cDNA and found that the enzyme belongs to the family of zinc metallopeptidases and contains the zinc-binding domain in its sequence. In addition, by an atomic absorption experiment we demonstrated that the recombinant enzyme contains a zinc ion, which we presume to be catalytic.

EXPERIMENTAL PROCEDURES

Materials—Various aminoacyl-NAs (Arg-, Bz-Arg-, Leu-, Ile-, Val-, Ala-, Pro-, Ser-, Tyr-, Phe-, Met-) were obtained from Sigma, and NAs (Lys-, His-, Asp-, Hyp-, Trp-, Gly-) were from Koch-Light Lab., Ltd. (Colnbrook, United Kingdom). Restriction and modifying enzymes were from Toyobo Co., Ltd. (Osaka, Japan) and Takara Co., Ltd. (Tokyo, Japan). All other reagents were of analytical grade and were purchased from Nakarai Tesque (Kyoto, Japan) and Daiichi Chemicals (Tokyo, Japan).

Protein and Enzyme Assay—Protein was usually measured by the method of Hartree (11). However, when the sample solution contained a reducing agent, the protein concentration was measured by the method of Ross and Schatz (12); and if the sample solution contained a microco amounted of protein, the method of Sargent (13) was used, with bovine serum albumin used as the standard.

Enzyme activity using Arg-NA as substrate was measured by a slight modification of the method of Hoppe et al. (14). The incubation mixture comprised 10 μmol of Tris-HCl buffer (pH 7.0) containing 15 μmol of NaCl, 0.04 μmol of substrate, and an appropriate amount of enzyme plus water to 200 μl. After incubation at 25 °C for 20 min, 500 μl of 1 M acetate buffer (pH 4.0) containing 10% Tween 20 and 200 μl of Fast Garnet GBC (0.8 mg/ml H₂O) were added to the reaction mixture. The absorbance of the resulting diazo dye was measured at 530 nm. When the reaction mixture contained a reducing agent, the reaction was stopped by adding 100 μl of 40% trichloroacetic acid; then the amount of β-naphthylamide liberated was estimated by the method of Goldbarg and Rutenburg (15). One unit of the enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of β-naphthylamide per min at 25 °C.

Cloning and Sequencing of the Rat Ap-B cDNA—For isolation of the rat liver cDNA, mRNA from rat (female Wistar) liver was obtained by use of a mRNA isolation kit; then the cDNA was constructed with a Uni-ZAP synthesis kit from Stratagene. From this library, 1.5 × 10⁶ plaques were screened immunologically with polyclonal rabbit anti-serum against the human placental Ap-B (described below). pBluescript phagemids containing cDNA inserts between EcoRI and XhoI sites were excised from the Uni-ZAP clones by use of ExAssist helper phage. VCSM13 helper phage were used for the single-stranded rescue procedure.

The nucleotide sequence was determined by the dideoxynucleotide chain termination reaction method (16) using overlapping subclones prepared from either M13 mp18 and/or M13 mp19 generated by digestion of the insert with appropriate restriction endonucleases and using the single-stranded pBluescript SK (-) phagemid. Specific oligomers were purchased from Sawaday Co., Ltd. (Tokyo, Japan) and used as primers to fill in remaining gaps in the sequence. Fig. 1 shows the restriction map and sequence strategy.

Purification of Enzyme from Human Placenta—The purification procedure was developed from that reported by Nagata et al. (17), with the following modification. Human placenta was obtained at normal full-term delivery. Immediately after delivery, the membranes and cord were dissected away, and the placenta was washed free from blood with cold 0.9% NaCl and cut into small pieces (311 g, wet weight). It was then
homogenized with an Ultra Turrax homogenizer (20,000 rpm, 3 min) in 8 volumes of 0.9% NaCl. The homogenate was centrifuged at 10,000 × g for 30 min, and the resulting supernatant was centrifuged at 55,000 × g for 60 min. The supernatant fluid was fractionated by ammonium sulfate precipitation (40–65% saturation) with solid ammonium sulfate. All following purification steps were carried out at 4 °C, if not otherwise specified. Each step used sodium phosphate buffer (10 mM, pH 7.5; molecular weight of the purified enzyme was determined by SDS-PAGE; as described by Laemmli (18). The purified enzyme or Escherichia coli cells were added to an equal volume of sample buffer containing 4% SDS and 2% 2-mercaptoethanol and boiled for 2 or 20 min, respectively. An appropriate amount of the purified enzyme or E. coli extract was used in each study. Uniform polycrylamide slab gel (10%) with a 5% stacking gel was run at room temperature until the bromphenol blue reached the bottom of the slab. The gel was then stained with Coomassie blue or used for Western blot analysis. Western Blot Analysis—The purified enzyme and crude cell extract were separated by SDS-PAGE on a 10% separating gel and transferred to nitrocellulose membranes (Bio-Rad) in a Bio-Rad Mini Trans-Blot apparatus. The membranes were blocked at room temperature in PBS, pH 7.5, containing 3% bovine serum albumin and 0.05% Tween 20. Rabbit anti-human Ap-B antiserum was diluted 1:3000 with the same buffer, and the membranes were incubated in it overnight at 4 °C. Subsequently, the membranes were washed three times in PBS containing 0.05% Tween 20 and incubated for 1 h with goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) diluted 1:2000 in blocking solution. After the membranes had been washed containing 0.05% Tween 20, the bands were visualized by use of an alkaline phosphatase color development kit (Bio-Rad). Purification of Recombinant Enzyme— Cultures of E. coli SOLI cells infected with the recombinant pbLuescript phagemid were grown at 37 °C to A600 = 0.2 in 1 liter of Luria-Bertani medium containing 50 mg of ampicillin. Then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1.0 mM, and the bacteria were incubated overnight. After centrifugation at 10,000 × g for 15 min, the cells were suspended in 70 ml of 0.9% NaCl and disrupted by ultrasonic treatment at 9 kHz for 1 min, repeated three times at intervals of 5 min. A clear supernatant fluid was obtained by centrifugation at 25,000 × g for 30 min, to which solid ammonium sulfate was added (65% saturation). The pellet obtained by centrifugation at 10,000 × g for 10 min was dissolved in buffer A and dialyzed against this same buffer. This solution (21.2 ml) was then applied to a DEAEE-Toyo column (2.8 × 3 cm) previously equilibrated with the same buffer. After the column had been washed with 130 ml of the same buffer, the enzyme was eluted with a linear NaCl gradient from 0 to 0.3 M in the buffer (flow rate, 174 ml/h). As in the case of the purification of the placental enzyme, the active enzyme fraction was then applied to a hydroxyapatite column (0.6 × 5 cm). After the hydroxyapatite column had been washed with 30 ml of buffer B, the enzyme was eluted with a linear phosphate gradient from 1 to 100 mM in buffer B (flow rate, 28 ml/h). The enzyme fractions were pooled, dialyzed against buffer A containing 50 mM NaCl and 1 mM mercaptoethanol, and then concentrated to 0.5 ml. This enzyme preparation was mixed with an equal volume of 50% glycerol and stored at –20 °C until used.

Results

Molecular Cloning of Rat Liver Ap-B—Ap-B was purified to homogeneity from human placenta, as summarized in Table I and was shown to give one main band and some minor ones by SDS-PAGE (Fig. 2A). Therefore, to obtain specific antibody, it was necessary to excise this main band for use as an immuno- gen. The enzyme hydrolized only Arg- and Lys-NA (activity ratio, 100:45), and the activity was inhibited by 1 mM bestatin, o-phenanthroline, and p-chloromercuribenzoic acid, which chemicals are known as inhibitors of Ap-B. The specific activity for hydrolysis of arginyl-NA, 14.3 units/mg of protein, was higher than the values for human (17), rat (4), and porcine (8) Ap-B reported previously.

A rat liver cDNA library was screened immunologically, and then five positive clones were isolated. To express the protein in E. coli, we excised the pbLuescript phagemids from these positive clones. Two of the five clones had a high Arg-NA-hydrolyzing activity, so we expected them to contain a full-length cDNA. The molecular mass of the insert cDNA and the nucleotide sequence of the 5′ region of the insert cDNA of both clones were the same; therefore, we concluded that the clones were identical.

The nucleotide sequence and the deduced amino acid sequence of the cDNA are shown in Fig. 3. The ATG codon

![Fig. 1. Schematic structure, restriction map, and sequencing strategy for Ap-B.](image-url)

**A** 5′- and 3′-noncoding regions; **B**, protein coding region; **arrows**, direction and extent of each sequence determination; **○**, synthesis primers; **E, EcoRI; A, Sau3AI; X, XhoI; bps, base pairs.

**SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)—** The purity and molecular weight of the purified enzyme were determined by SDS-PAGE as described by Laemmli (18). The purified enzyme or E. coli cells were added to an equal volume of sample buffer containing 4% SDS and 2% 2-mercaptoethanol and boiled for 2 or 20 min, respectively. An appropriate amount of the purified enzyme or E. coli extract was used in each study. Uniform polycrylamide slab gel (10%) with a 5% stacking gel was run at room temperature until the bromphenol blue reached the bottom of the slab. The gel was then stained with Coomassie blue or used for Western blot analysis.

**Western Blot Analysis—** The purified enzyme and crude cell extract were separated by SDS-PAGE on a 10% separating gel and transferred to nitrocellulose membranes (Bio-Rad) in a Bio-Rad Mini Trans-Blot apparatus. The membranes were blocked at room temperature in PBS, pH 7.5, containing 3% bovine serum albumin and 0.05% Tween 20. Rabbit anti-human Ap-B antiserum was diluted 1:3000 with the same buffer, and the membranes were incubated in it overnight at 4 °C. Subsequently, the membranes were washed three times in PBS containing 0.05% Tween 20 and incubated for 1 h with goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) diluted 1:2000 in blocking solution. After the membranes had been washed containing 0.05% Tween 20, the bands were visualized by use of an alkaline phosphatase color development kit (Bio-Rad). Purification of Recombinant Enzyme— Cultures of E. coli SOLI cells infected with the recombinant pbLuescript phagemid were grown at 37 °C to A600 = 0.2 in 1 liter of Luria-Bertani medium containing 50 mg of ampicillin. Then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1.0 mM, and the bacteria were incubated overnight. After centrifugation at 10,000 × g for 15 min, the cells were suspended in 70 ml of 0.9% NaCl and disrupted by ultrasonic treatment at 9 kHz for 1 min, repeated three times at intervals of 5 min. A clear supernatant fluid was obtained by centrifugation at 25,000 × g for 30 min, to which solid ammonium sulfate was added (65% saturation). The pellet obtained by centrifugation at 10,000 × g for 10 min was dissolved in buffer A and dialyzed against this same buffer. This solution (21.2 ml) was then applied to a DEAEE-Toyo column (2.8 × 3 cm) previously equilibrated with the same buffer. After the column had been washed with 130 ml of the same buffer, the enzyme was eluted with a linear NaCl gradient from 0 to 0.3 M in the buffer (flow rate, 174 ml/h). As in the case of the purification of the placental enzyme, the active enzyme fraction was then applied to a hydroxyapatite column (0.6 × 5 cm). After the hydroxyapatite column had been washed with 30 ml of buffer B, the enzyme was eluted with a linear phosphate gradient from 1 to 100 mM in buffer B (flow rate, 28 ml/h). The enzyme fractions were pooled, dialyzed against buffer A containing 50 mM NaCl and 1 mM mercaptoethanol, and then concentrated to 0.5 ml. This enzyme preparation was mixed with an equal volume of 50% glycerol and stored at –20 °C until used.

**Zinc Content of Recombinant Enzyme—** Zinc determinations were performed by electrothermal atomic absorption spectrometry with a Zeeman atomic absorption spectrometer SM-30(Shimazu Co., Ltd., Tokyo, Japan).

Absorbance peak heights were measured at 307.6 nm. For the zinc determination, the enzyme was applied to a Nickel column (containing Sephadex G-50; Pharmacia Biotech Inc.) and eluted with distilled water. Then the eluted solution was divided into three parts for analysis of zinc and protein and for determination of amino acid composition. The sample for zinc analysis was lyophilized, dissolved in 0.01% metal-free nitric acid, and appropriately diluted. Ten-microliter samples were loaded into pyrolytically coated graphite tubes. All values shown are averages of duplicate determinations on four different dilutions of sample. Zinc concentrations were quantified by comparing unknowns with zinc standard solutions (0, 50, 75, and 100 ng; Wako Pure Chemical Ind., Ltd.).

**Amino Acid Composition of Recombinant Protein—** Amino acid composition of the sample described above was determined after hydrolysis under vacuum in 6 M HCl vapors at 110 °C for 24, 48, and 72 h, followed by precolumn derivatization with phenylisothiocyanate (Waters Associates, PICO-TAG method) and reversed-phase high-performance liquid chromatography.
human placenta (enzyme following electrophoresis. Loaded onto a standard 10% gel and stained with Coomassie Blue.

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domains (30), regions II and III shown in Fig. 4, were not conserved.

Purification and Properties of Recombinant Ap-B—The recombinant enzyme (1.33 mg) was obtained from extracts of E. coli SORL cells (from 1 liter of medium) infected with the pBluescript phagemid containing Ap-B cDNA. It had a specific activity of 27.0 units/mg protein, which corresponded to 117-

fold purification from ammonium sulfate fraction with a recovery of 57%.

The relative rate of hydrolysis of various aminoacyl-NAs by the recombinant enzyme was approximately the same as that of human placental Ap-B. (activity ratio: Arg:Lys = 100:44.4). The other synthetic substrates were not hydrolyzed at all. The pH optimum (pH 7.4) and the molecular mass did not differ significantly between the two enzymes. $K_v$ values (mean ± S.D.) for Arg-NA of the purified enzymes were $(1.4 ± 0.3) \times 10^{-4} \text{ M}$ (placental) and $(2.3 ± 0.2) \times 10^{-4} \text{ M}$ (recombinant). The catalytic efficiency ($K_{cat}/K_v$ (M$^{-1}$ s$^{-1}$)) of the two enzymes toward Arg-NA was almost the same order of magnitude: placental, 340,000; and recombinant, 460,000. $K_v$ values (mean ± S.D.) of the recombinant enzyme were $(5.4 ± 0.3) \times 10^{-8} \text{ M}$ for bestatin (competitive inhibition), $(2.2 ± 0.1) \times 10^{-4} \text{ M}$ for o-phenanthroline (mixed type noncompetitive inhibition), and $(4.9 ± 2.2) \times 10^{-8} \text{ M}$ for p-chloromercuribenzoic acid (uncompetitive inhibition) as determined from a Lineweaver-Burk plot by a linear regression program. The modes of inhibition of the recombinant enzyme activity were the same as those previously reported for other mammalian enzymes (7, 8).

The zinc content of the recombinant enzyme, determined by atomic absorption spectrometry, was $1.14 ± 0.28$ (mean ± S.D.) and $1.16 ± 0.12$ (mean ± S.D.) mol zinc per mol of enzyme as calculated from protein assay and amino acid composition, respectively. Thus we conclude that Ap-B has 1 mol of zinc per mol of enzyme.

DISCUSSION

We were successful in cloning the cDNA encoding rat Ap-B. The deduced amino acid sequence contains the HEXXX consensus sequence (residues 324–348) with a second glutamic acid separated by 18 amino acids, which is the structural signature of members of the zinc metallopeptidase family (20, 21). Search of the Swiss Prot (EMBL) protein sequence data base with the primary structure of rat liver Ap-B showed significant similarity to the leukotriene-A$\gamma$ hydrolase of rats (36.5% identity over 598 amino acids) (22), humans (37.2% identity over 596 amino acids) (23), mice (37.3% identity over 598 amino acids) (24), and guinea pigs (38.1% identity over 535 amino acids) (25). Lower but highly significant levels of amino acid sequence identity were found between Ap-B and the Ap-N family (21). The homology was especially striking in the region (amino acid residues 151–353) containing the consensus zinc-binding motif, and the identity between Ap-B and rat leukotriene-A$\gamma$ hydrolase was 55.2% (Fig. 4). However, when the amino acid sequence of Ap-B was compared with that of other metallopeptidases (yeast Ap, mouse pre-B-cell antigen, human Ap-N, and rat zinc Ap), the consensus sequences of zinc-binding motifs (21) were not conserved.

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Ap-B is 72,545 Da, which is approximately the same as the molecular mass of human enzymes from: skeletal muscle, 72,000 Da (33); brain, 70,000 Da (34); chronic myeloid cell, 73,000 Da (35); T lymphocytes, 72,000 Da (36); and rat testis enzyme, 72,000 Da (37). Belhacene et al. (36) reported that the enzyme in human T lymphocytes was sensitive to proteolysis during purification and storage. In line with this, we found that whole E. coli lysates containing recombinant Ap-B showed three bands by Western blot analysis. One band had the same electrophoretic mobility as the purified enzyme, but the other two bands were estimated to have a lower molecular mass, which we supposed to be degraded enzyme protein (Fig. 2B).

Therefore, the purified Ap-Bs from rat muscle (38) and porcine liver (39), which had a molecular mass estimated to be 72,000 Da, may have been degraded. Because the molecular masses of Ap-B isolated from rat liver (4), rat blood cells (39), porcine muscle (40), human erythrocytes (41), human skeletal muscle (42), and human placenta (18) are >73,000 Da, another translation initiation site might exist upstream in the 5′-end of Ap-B cDNA.

Ap-B from human erythrocytes has relatively weak endopeptidase activity in hydrolyzing the internal Gly4–Phe5 bond of bradykinin (43, 44). Similar enzymes are reported to exist in skeletal muscle (33) and brain (34), but they hydrolyze a different internal bond (Phe5–Ser6) in the case of bradykinin. If this is true, Ap-B would seem to be a bifunctional enzyme like previously reported peptidases (45–47). Recently, the endopeptidase activity in a preparation of purified rat testis Ap-B (37) was identified as contaminating thimet oligopeptidase (48) (EC 3.4.24.15) by an inhibition experiment utilizing phosphodiester O3 (49). Therefore, using bradykinin and neurotensin as substrates, we tested the endopeptidase activity of our recombinant enzyme. Both bioactive peptides were split at one internal bond site (data not shown). Although the enzyme action was completely inhibited by 1 mM o-phenanthroline, the site of hydrolysis of two peptides was different from that of the thimet oligopeptidase (50). The endopeptidase activity in the recombinant enzyme is probably due to the contamination of some other metalloendopeptidase. However, at present, we do not know whether Ap-B has its own endopeptidase activity or not. Further experiments using site-directed mutagenesis of the zinc-binding domain, as done for leukotriene-A4 hydrolase (51), and identification of the endogenous physiological substrate, as in the case of T lymphocyte (36), remain to be accomplished to define the physiological function of Ap-B.
FIG. 4. Ap and leukotriene-A₄ hydrolase (LKHA) sequence alignment. The deduced amino acid sequence of rat Ap-B (amino acids 151–353) is aligned with the sequences of human Ap-A (30) (amino acid 115–353) is aligned with the sequences of human Ap-A (30) (amino acid 115–353) and conserved glutamic acid are in boldface. Highly conserved regions of several Aps are designated by underlines and Roman numerals.

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