Aminopeptidase B is structurally related to leukotriene-A₄ hydrolase but is not a bifunctional enzyme with epoxide hydrolase activity

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Aminopeptidase B (Ap B; EC 3.4.11.6) is a zinc-binding protein that contains the consensus sequence HEXXH₁₈E (324–347), conserved among the M1 family of metalloproteases. To determine if these putative zinc-binding residues (His³²⁴, His³²⁸ and Glu³⁴⁷) and the active-site Glu³²⁵ are essential for the enzyme activity, we replaced the histidines with tyrosines and the glutamic acid residues with alanines using site-directed mutagenesis. The cDNAs were expressed in *Escherichia coli*, and the resulting recombinant proteins, named H324Y, E325A, H328Y and E347A, were purified to apparent homogeneity. None of the expressed mutated proteins showed aminopeptidase activity. The E325A enzyme contained 1 mol of zinc per mol of protein, and the other three mutants, H324Y, H328Y and E347A, did not contain significant amounts of zinc, as determined by atomic absorption spectrometry. From sequence-homology searches,

Ap B is known to be closely related to leukotriene (LT)-A₄ hydrolase (EC 3.3.2.6). We examined human placental Ap B and recombinant rat Ap B, both of which had been purified previously [Fukasawa, Fukasawa, Kanai, Fujii and Harada (1996) *J. Biol. Chem.* **271**, 30731–30735], to determine whether or not they had epoxide hydrolase activities. However, neither enzyme hydrolysed LTA₄ into LTB₄. We then replaced some amino acids in the domain of the rat enzyme similar to the LTA₄-binding site of LTA₄ hydrolase. However, these mutants, Y408F, N409S and NE409–410SS also did not possess any epoxide hydrolase activity. We concluded that Ap B is an M1-family zinc metalloprotease without epoxide hydrolase activity.

Key words: catalytic site, gluzincin, metal binding ligands, site-directed mutagenesis.

INTRODUCTION

Previously we cloned the full-length cDNA coding for rat aminopeptidase (Ap) B and expressed the protein in *E. coli* [1]. Ap B revealed the presence of the consensus sequence HEXXH, found in the zinc metalloprotease family [2–4], and was classified as a ‘zincin’ [5]. The X-ray crystal structure of thermolysin revealed that the two histidine residues and the glutamic acid residue in this motif are involved in zinc co-ordination and catalytic activity, respectively [6]. Ap B is classified as a member of the M1 family [2] or Ap family [7]. There are several Aps (Ap N and Ap A from mammals, Ap N from *E. coli*, AAP1 and APE2 from *Saccharomyces cerevisiae*, lap Ap from *Lactococcus lactis*, and lysyl Ap from *L. delbrückii*), as well as leukotriene (LT)-A₄ hydrolase, that are members of the Ap family [7]. Most of these enzymes are predicted to bind zinc because they conserve the consensus sequence HEXXH₁₈E. These predictions have been proven to be true for LTA₄ hydrolase [8–10] and Ap A [11,12] by site-directed-mutagenesis studies.

From sequence-homology searches, Ap B is known to be the most closely related to LTA₄ hydrolase among the members of this family. It has been shown by several groups of investigators, including ours [13–15], that LTA₄ hydrolase is a bifunctional enzyme possessing peptidase activity as well as epoxide hydrolase activity.

In the present study, three putative zinc-binding residues and a catalytic glutamic acid residue in the consensus sequence of Ap B were demonstrated by site-directed mutagenesis. Moreover, in order to clarify whether Ap B is a bifunctional enzyme with epoxide hydrolase activity or not, we assayed the epoxide hydrolase activities of human and rat Ap Bs.

MATERIALS AND METHODS

Materials

Arg-β-naphthylamide (Arg-NA) was obtained from Sigma, and Arg-4-methyl-coumaryl-7-amide (Arg-MCA) and amastatin were from Peptide Institute (Osaka, Japan). Restriction and modifying enzymes were from Toyobo Co. (Osaka, Japan) and Takara Co. (Tokyo, Japan), respectively. Oligonucleotides were synthesized by Sawaday Co. (Tokyo, Japan). All other reagents were of analytical grade and were purchased from Nakarai Tesque (Kyoto, Japan) and Daiichi Chemicals (Tokyo, Japan).

Site-directed mutagenesis and expression in *E. coli*

The expression plasmid (pBluescript phagemid SK) containing the cDNA encoding rat Ap B was constructed previously [1]. Mutagenesis was performed on the cDNA using the Chameleon double-stranded site-directed mutagenesis kit from Stratagene, according to the manufacturer’s recommended protocol. The plasmid containing the cDNA was heat-denatured, and *SpeI* or *NotI* selection primers and appropriate mutagenic primers (Table 1) were annealed to one of the strands. Both of these primers were extended around the plasmid and ligated to create the new mutant DNA strand. After restriction digestion with *SpeI* or *NotI* to linearize the strand, the remaining circular (mutated) plasmid was transformed into a repair-deficient mut S *E. coli* strain (XLmut S competent cells) and the transformed cells were selected by growth on ampicillin. Then the cells were grown in liquid culture and plasmid DNA was isolated. After a second restriction digestion with *SpeI* or *NotI*, the resultant DNA was

Abbreviations used: Ap, aminopeptidase; LT, leukotriene; NA, β-naphthylamide; MCA, 4-methyl-coumaryl-7-amide; AA, amino acid.

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Table 1 Primers used for mutagenesis of rat Ap B cDNA

Mutated bases are underlined.

Mutation	Nucleotide sequence (5' → 3')	Numbering
Selection primer		
SpeI site change	CCGCTCTAGAACATGTGGATCCCC	
NotI site change	CCGCGGTGGCGCCGCTCTAGAAC	
Mutagenic primer		
His ³²⁴ → Tyr	CGACGTCATCATCTACGAGATCTCC	957–981
Glu ³²⁵ → Ala	CATCATCCACGCGATCTCCAC	963–984
Glu ³²⁵ → Asp	CATCATCCACGAGATCTCCAC	963–984
Ser ³²⁷ → Ala	CCAGGAGATCGCCACAGTTGG	969–990
His ³²⁸ → Tyr	CACGAGATCTCTACAGTTGGTTG	970–994
Glu ³⁴⁷ → Ala	GCTCAATGCAGGCTTC	1032–1047
Tyr ⁴⁰⁸ → Phe	CAGATGACACCTTCAATGAAACC	2011–2033
Asn ⁴⁰⁹ → Ser	GATGACACCTACTCTGAAACCCCC	2013–2036
Asn ⁴⁰⁹ –Glu ⁴¹⁰ → Ser–Ser	CACCTACTCTTCAACCCCTAC	2018–2039

used to transform *E. coli* SOLR competent cells. The single colony was grown in liquid culture for identifying the expressed protein by SDS/PAGE, and the single-stranded DNA was isolated by infecting the bacteria with VCSM 13 helper phages for sequencing. Sequence analysis of the entire cDNA inserts confirmed that no alteration of the protein primary structures, other than the desired mutations, had occurred. The recombinant single-stranded plasmids were sequenced by the dideoxy chain-termination procedure [16], using appropriate synthetic oligonucleotides.

Expression and purification of recombinant Ap B mutants

Mutated proteins were expressed and then purified essentially as described previously [1]. The procedure involved precipitations, anion-exchange, hydroxyapatite, hydrophobic interaction and gel-filtration chromatographies. In the case of inactive mutants, the expressed proteins were detected by dot-blot immunoassay using rabbit anti-human Ap B antiserum in each step. Three different clones were separately studied for each mutant.

SDS/PAGE and Western-blot analysis

The purity of the recombinant enzymes and the expression of the recombinant proteins in the *E. coli* cells were confirmed by SDS/PAGE as described by Laemmli [17]. The purified enzymes and the recombinant cells were boiled for 5 and 20 min, respectively, in Laemmli sample buffer [0.0625 M Tris/HCl (pH 6.8)/2% SDS/10% glycerol/5% 2-mercaptoethanol/0.001% Bromophenol Blue] and loaded on to a polyacrylamide gel (10% separating gel/5% stacking gel). Electrophoresis was performed for each sample in duplicate: one gel was stained with Coomassie Brilliant Blue to visualize the protein bands, and the other was used for Western-blot analysis. The separated proteins were then transferred on to supported nitrocellulose membrane (Bio-Rad) in a Bio-Rad Mini Trans-Blot apparatus. The membrane was blocked at room temperature in PBS containing 3% BSA and 0.05% Tween 20. Subsequently, the membrane was incubated overnight at 4 °C with a rabbit antiserum raised against human Ap B, as described previously [1], diluted 1:3000 with the same buffer. After having been washed with PBS containing 0.05% Tween 20, the membrane was exposed to a second antibody, goat anti-rabbit IgG conjugated with alkaline phosphatase, diluted 1:2000 in blocking solution. The membrane was washed three

times in PBS containing 0.05% Tween 20, and the immuno-reactive bands were detected by use of an alkaline phosphatase colour-development kit (Bio-Rad).

Protein and enzyme assay

The protein of the cell suspension was measured by the method of Hartree [18], and the protein content discussed in the following steps was measured according to Bradford [19] with BSA used as the standard.

Enzyme activity measured with Arg-NA as the substrate was described previously [1]. Enzyme activity with Arg-MCA as the substrate was measured fluorometrically by monitoring the rate of 7-amino-4-methylcoumarin formation at 460 nm with excitation at 380 nm. Enzyme activity (1 unit) was defined as the amount of enzyme catalysing the formation of 1 µmol of β-naphthylamine or 7-amino-4-methylcoumarin per min at 37 °C.

Activity of LTA₄ hydrolase was measured according to a method reported previously [20] with minor modification. The standard assay mixture contained 0.1 M Tris/HCl buffer (pH 7.8), 2 mg/ml BSA and the sample in a total volume of 50 µl. After preincubation for 3 min at 37 °C, 1 µl of LTA₄ in ethanol solution containing lithium hydroxide was added to give a final concentration of 30 µM. After a 3 or 15 min incubation at 37 °C, the reaction was terminated by the addition of 100 µl of a stop solution [acetonitrile/methanol/acetic acid, 150:50:3 (by vol.), containing 0.3 nmol of prostaglandin B₂ as an internal standard]. The mixture was kept at –25 °C for 30 min and then centrifuged at 10000 g for 10 min. An aliquot (50 µl) of the supernatant was analysed by reversed-phase HPLC. The column (TSK ODS-80TM, 5 µm, 0.46 cm × 15 cm; Toyosoda, Tokyo) was eluted with solvent I [acetonitrile/H₂O/acetic acid, 50:50:0.1 (by vol.), containing 0.02% Na₂EDTA (pH 5.6) with ammonium water) at a flow rate of 1 ml/min, or solvent II [acetonitrile/methanol/H₂O/acetic acid, 37.5:12.5:50:0.1 (by vol.), containing 0.01% Na₂EDTA] at a flow rate of 1.5 ml/min. The absorbance at 270 nm was monitored, and the amount of LTB₄ formed was calculated from the peak-area ratio of LTB₄/prostaglandin B₂. LTB₄, with the typical triplet UV absorbance showing three maximum peaks of 260, 270 and 281 nm, was eluted with solvent I at 6.88 min, and with solvent II at 17.5 min. The calculated LTB₄ amounts with both solvent systems were essentially the same. Enzymic production of LTB₄ was calculated from total production of LTB₄ minus its non-enzymic production. Also, the HPLC fractions from 17.0 to 18.0 min with solvent II were evaporated under N₂ gas, and analysed using an Enzyme Immunoassay Kit (Cayman Chemical Co.).

Zinc content of mutated proteins

Zinc determinations were performed by electrothermal atomic absorption spectrometry using 300 µg of each purified protein according to a method described previously [1].

RESULTS

Expression and purification of mutated proteins

E. coli cells harbouring the mutated recombinants were identified, and the expressed proteins were detected by staining with Coomassie Brilliant Blue and by their immunoreactivity after SDS/PAGE. The values for total protein in all cultures were approximately the same, and the mean value was 439 ± 32 mg per 1 litre culture. All mutant cells expressed almost the same amounts of recombinant proteins, and these proteins showed the same mobility by SDS/PAGE (results not shown). The peptidase

Table 2 Ap activities of *E. coli* cells transformed with Ap B mutants

Before an enzyme assay, the cell suspensions were preincubated with 0.1 mM amastatin at 37 °C for 10 min to inhibit the leucine Ap derived from *E. coli* cells. The enzyme assay was carried out with Arg-NA as a substrate. N.D., not detectable.

	Ap activity (m-units/mg of protein; means $\pm$ S.D.)	Percentage of wild type
Wild-type	58.3 $\pm$ 15.4	100
H324Y	N.D.	—
E325A	N.D.	—
E325D	N.D.	—
S327A	84.7 $\pm$ 24.2	150
H328Y	N.D.	—
E347A	N.D.	—
Y408F	47.2 $\pm$ 3.48	81
N409S	57.1 $\pm$ 2.18	98
NE409–410SS	9.89 $\pm$ 0.29	17

Table 3 Zinc content of the rat Ap B mutants

The wild-type enzyme was analysed previously [1].

Enzyme	Batch no.	Zinc content	
		Mol/mol of protein	Means $\pm$ S.D.
Wild type			1.14 $\pm$ 0.28
H324Y	1	0.05	
	2	0.11	
	3	0.07	0.077 $\pm$ 0.025
E325A	1	0.97	
	2	0.90	
	3	1.12	0.997 $\pm$ 0.092
H328Y	1	0.15	
	2	0.04	
	3	0.08	0.090 $\pm$ 0.045
E347A	1	0.10	
	2	0.08	
	3	0.07	0.083 $\pm$ 0.012

Figure 1 SDS/PAGE of purified mutated and wild-type recombinant rat Ap Bs

(A) The mutated proteins H324Y (lane 2), E325A (lane 3), H328Y (lane 4), E347A (lane 5), Y408F (lane 6), N409S (lane 7) and NE409–410SS (lane 8), and the wild-type Ap B (lane 1) (5.0 μ g each) were electrophoresed on a standard 10% gel and stained with Coomassie Brilliant Blue. Mr, molecular-mass markers (kDa). (B) Western-blot analysis of the mutated proteins H324Y (lane 12), E325A (lane 13), H328Y (lane 14), E347A (lane 15), Y408F (lane 16), N409S (lane 17) and NE409–410SS (lane 18), and the wild-type Ap B (lane 11) (1.0 μ g each).

activities of the cells with the mutated plasmids are shown in Table 2. The mutants H324Y, E325A, E325D, H328Y and E347A were practically devoid of peptidase activity. In contrast, mutagenetic replacement at position 327 (S327A) resulted in 150 % of the peptidase activity of the wild-type enzyme.

Moreover, to determine the zinc content of the mutated proteins H324Y, E325A, H328Y and E347A that were devoid of peptidase activity, and to study the enzymic properties of the mutated proteins Y408F, N409S and NE409–410SS that retained the peptidase activity, we purified these mutated proteins to apparent homogeneity (Figure 1A). These proteins, which behaved like the wild-type enzyme in all purification steps, were also identified as a single protein by Western-blot analysis (Figure 1B). Finally, 0.5–1.0 mg of each purified protein was obtained from 1 litre of *E. coli* cell culture.

Zinc contents and peptidase activities of mutated proteins (H324Y, E325A, H328Y and E347A)

The zinc contents of the mutated proteins were determined by atomic absorption spectrometry (Table 3). The mutant E325A contained about 1 mol of zinc per mol of protein, the same as the wild-type Ap B. On the other hand, mutants H324Y, H328Y and E347A, in which one of the putative zinc-binding residues had been replaced, contained 0.077, 0.090 and 0.083 mol of zinc per mol of protein, respectively. Also, we could not detect any significant peptidase activities in any of these three mutants after purification.

Epoxide hydrolase activities of Ap B from human placenta, recombinant rat Ap B, and the mutants Y408F, N409S and NE409–410SS

The Ap B from human placenta and the recombinant rat Ap B were purified to homogeneity previously [1]. For the epoxide hydrolase activity assay, 2 μ g of each purified protein were used. We could not detect any enzymic conversion of LTA₄ to LTB₄ in either human or rat Ap B with the HPLC method (Figures 2A, 2B and 2E) and with the LTB₄ enzyme immunoassay. The three

Figure 2 Reversed-phase HPLC analysis of LTA₄ hydrolase activity

The purified enzymes were assayed separately and analysed as described in the Materials and methods section. Human placental Ap B (**A**), recombinant rat Ap B (**B**), mutated enzyme Y408F (**C**), the positive control (10000-g supernatant of guinea pig lung; **D**), and no enzyme (**E**) were examined. Peaks I and II correspond to prostaglandin B₂ and the epimers at C¹² of Δ⁶-trans-LTB₄, respectively. Arrows show the position of LTB₄.

Table 4 Kinetic constants for peptidase activity

Protein	Substrate	K_m (means $\pm$ S.D., mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($10^4 M^{-1} \cdot s^{-1}$)
Wild type	Arg-NA	0.110 ± 0.009	53.1	482
	Arg-MCA	0.132 ± 0.001	57.7	437
Y408F	Arg-NA	0.317 ± 0.016	13.9	43.8
N409S	Arg-NA	0.222 ± 0.006	47.1	212
NE409–410SS	Arg-MCA	0.098 ± 0.011	5.95	60.7

mutants Y408F, N409S and NE409–410SS, in which one or two amino acids in the domain similar to the LTA₄-binding site of LTA₄ hydrolase were replaced, also did not hydrolyse LTA₄ to LTB₄. The HPLC chromatogram for Y408F is shown in Figure 2(C) as a representative result, but these findings were also confirmed using both HPLC analysis and LTB₄ enzyme immunoassay. The supernatant of guinea pig lung homogenate (10 μ l) was also assayed for LTA₄ hydrolase activity as a positive control, as shown in Figure 2D.

Kinetic constants for hydrolysing Arg-NA or Arg-MCA of the mutants Y408F, N409S and NE409–410SS

The kinetic constants K_m , k_{cat} and k_{cat}/K_m for the peptidolysis were analysed for the mutants Y408F, N409S and NE409–410SS and the wild-type enzyme (Table 4). These three mutations decreased both k_{cat} and k_{cat}/K_m . The k_{cat}/K_m values of the mutants Y408F, N409S and NE409–410SS were reduced by 9, 44 and 14 % of that of the wild-type enzyme, respectively.

DISCUSSION

In this mutagenesis study, the mutated proteins H324Y, H328Y and E347A contained insignificant amounts of zinc (Table 3) and had no peptidase activity (Table 2). On the other hand, the mutated protein E325A contained one mol of zinc per mol of protein; however, it had no peptidase activity. Nevertheless, the behaviour of the mutated proteins in all purification steps was approximately the same as that of the wild-type recombinant Ap B. Also, the mutant S327A, which was tested for the effects of mutagenetic replacement at residue 327, retained the peptidase activity. Thus we concluded that His³²⁴, His³²⁸ and Glu³⁴⁷ located in the HEXXH₁₈E motif (324–347) of Ap B constitute three zinc-binding residues and that Glu³²⁵ plays a crucial role in catalysis.

Ap B is the most closely related to LTA₄ hydrolase of the members of the Ap family, as shown by the alignment of homologous-region sequences of some members of the M1 family (Figure 3). But the human and recombinant rat Ap Bs did not hydrolyse LTA₄ to LTB₄ (Figure 2). The Tyr³⁷⁸ residue of LTA₄ hydrolase was shown to bind to LTA₄ to form the activated enzyme–substrate complex that can either break down to the free enzyme and LTB₄ or result in covalent-bond formation and inactivation [21]. In that study, the epoxide hydrolase activity of the mutant Y378F of LTA₄ hydrolase was found to increase (178 % of the wild-type enzyme). Therefore, we replaced Tyr⁴⁰⁸ of Ap B with Phe, and the adjacent Asn or Asn–Glu at positions 409 or 409–410 by Ser or Ser–Ser, which are more closely related to the LTA₄-binding domain of LTA₄ hydrolase than the non-mutated Ap B. However, none of these mutants, Y408F, N409S or NE409–410SS, exhibited significant epoxide hydrolase activity (Figure 2).

On the other hand, these three mutations in the domain similar to the LTA₄-binding site of LTA₄ hydrolase exhibited a reduction

Figure 3 Sequence alignment of Ap B, human LTA₄ hydrolase (LTA₄) [28], murine Ap A [29], and human Ap N [30]

I (bold sequence), the homologous region containing consensus zinc-binding sequence; II (bold sequence), the homologous region containing the LTA₄-binding residue of LTA₄ hydrolase. Amino acids in LTA₄ hydrolase, Ap A and Ap N identical to those of Ap B are indicated by asterisks, and several gaps are inserted into the sequences as bars. ●, Putative zinc-binding site; ○, putative active site for peptidolysis; ▲, LTA₄-binding residue of LTA₄ hydrolase. Mutated residues are underlined.

of peptidase activity (Table 4), so Tyr⁴⁰⁸, Asn⁴⁰⁹ and/or Glu⁴¹⁰ in this domain may be involved with peptidolysis. The Y408F mutation of Ap B decreased the k_{cat} and k_{cat}/K_m values to approximately same level as the Y378F mutant of LTA₄ hydrolase [21]. However, the hydroxyl group of the Ser³⁷⁹ of LTA₄ hydrolase was supposed to have some function for peptidolysis based on the peptidase activity of the mutant S379A being substantially reduced [21]. On the contrary, the N409S and NE409–410SS mutations of Ap B reduced the peptidase activity (Table 4). This difference is not contradictory to the difference in substrate specificity between Ap B and LTA₄ hydrolase, described below.

Ap B is a chloride-activated metalloenzyme that is widely distributed in mammalian tissues [22–24] and is specific for the liberation of arginyl and lysyl residues from peptides and synthetic substrates [amino acid (AA)-NA, AA-*p*-nitroanilide or AA-MCA] [24], but LTA₄ hydrolase is not so specific for N-terminal basic amino acids of peptides or AA-*p*-nitroanilide, but is rather more specific for tripeptides [25]. Ap B and LTA₄ hydrolase are both N-terminal exopeptidases, but their substrate specificities are different. It is therefore reasonable that Ap B and LTA₄ hydrolase would have different structures to account for their substrate specificities for peptidolysis.

From this study, we conclude that both catalytic domains of Ap B and LTA₄ hydrolase for peptidolysis have the same consensus zinc-binding domain (HEXXHX₁₈E). Cadel et al. [26] also obtained several clones containing a partial cDNA coding for rat Ap B and a linking nucleotide sequence of a 2.2 kb cDNA. In that study, they insisted that the Ap B purified from rat testis hydrolysed LTA₄ to LTB₄ by means of enzyme immunoassay and presumed that Ap B was a bifunctional enzyme, based on the strong resemblance of secondary structure between them. But *Caenorhabditis elegans* Ap, which is more closely related to LTA₄ hydrolase (45% identity at the amino acid level) [27] than to Ap B (37% identity at the amino acid level), has no LTA₄ hydrolase activity using HPLC and radioimmunoassay for LTB₄. This supports our findings that Ap Bs from rat and human, and the mutated rat Ap Bs, have no epoxide hydrolase activity. Ap B appears to have evolved as a different functional enzyme to LTA₄ hydrolase.

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