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Role of glutamine-169 in the substrate recognition of human aminopeptidase B

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ABSTRACT

Background: Aminopeptidase B (EC 3.4.11.6, APB) preferentially hydrolyzes N-terminal basic amino acids of synthetic and peptide substrates. APB is involved in the production and maturation of peptide hormones and neurotransmitters such as miniglucagon, cholecystokinin and enkephalin by cleaving N-terminal basic amino acids in extended precursor proteins. Therefore, the specificity for basic amino acids is crucial for the biological function of APB.

Methods: Site-directed mutagenesis and molecular modeling of the S1 site were used to identify amino acid residues of the human APB responsible for the basic amino acid preference and enzymatic efficiency.

Results: Substitution of Gln169 with Asn caused a significant decrease in hydrolytic activity toward the fluorescent substrate Lys-4-methylcoumaryl-7-amide (MCA). Substantial retardation of enzyme activity was observed toward Arg-MCA and substitution with Glu caused complete loss of enzymatic activity of APB. Substitution with Asn led to an increase in IC_{50} values of inhibitors that interact with the catalytic pocket of APB. The EC_{50} value of chloride ion binding was also found to increase with the Asn mutant. Gln169 was required for maximal cleavage of the peptide substrates. Molecular modeling suggested that interaction of Gln169 with the N-terminal Arg residue of the substrate could be bridged by a chloride anion.

Conclusion: Gln169 is crucial for obtaining optimal enzymatic activity and the unique basic amino acid preference of APB *via* maintaining the appropriate catalytic pocket structure and thus for its function as a processing enzyme of peptide hormones and neurotransmitters.

1. Introduction

Aminopeptidases hydrolyze N-terminal amino acids of proteins or peptide substrates [1]. Among them, the M1 family of zinc aminopeptidases shares GXMEN and $HEXXH(X)_{18}E$ motifs essential for enzymatic activity. This family, which comprises 11 human enzymes, plays important roles in several pathophysiological processes including angiogenesis, cell cycle regulation, reproduction, memory retention, blood pressure regulation and antigen presentation to major histocompatibility complex class I molecules [2].

Aminopeptidase B (EC 3.4.11.6, APB) is a monomeric secretory protein first detected in several rat tissues which preferentially removes basic amino acids (Arg and Lys) from the N-terminus of several peptide and fluorogenic substrates [3]. Characterization of the enzymatic properties of APB showed that the hydrolytic activity of the enzyme was enhanced in the presence of physiological concentrations of chloride anions [4].

cDNA cloning of the rat APB revealed that the enzyme belongs to the M1 family and is closely related to leukotriene A_4 hydrolase (LTA4H), which converts LTA_4 to LTB_4 [5,6]. LTA4H contains both GXMEN and $HEXXH(X)_{18}E$ motifs and also has aminopeptidase activity [7]. Similarly, it was reported that human APB also shows hydrolytic activity toward LTA_4, suggesting that the enzyme plays a role in certain inflammatory processes; however, another group could not detect this activity [6,8].

Since APB shows preference for basic amino acids, this enzyme has been suggested to be involved in the proteolytic processing and maturation of peptide hormones and neurotransmitters [3,9]. APB has been reported to be involved in the generation of active peptides through cleavage of extended N-terminal Arg and Lys residues in intermediate propeptides produced by endopeptidases such as cathepsin L and N-arginine dibasic convertase (NRD)/nardilysin [9–11]. Moreover, APB co-localizes with cathepsin L in neuropeptide-containing secretary

Abbreviations: APB, aminopeptidase B; IC_{50}, half maximal (50%) inhibitory concentration; EC_{50}, half maximal (50%) effective concentration; ERAP, endoplasmic reticulum aminopeptidase; LTA4H, leukotriene A_4 hydrolase; MCA, 4-methylcoumaryl-7-amide; NRD, N-arginine dibasic convertase

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vesicles [9]. Given that the hydrolytic activity of APB toward Arg and Lys residues is essential for processing and maturation of hormones and neurotransmitters, it is important to elucidate the structural aspects underlying APB activity. In this context, Fukasawa et al. reported that replacement of Asp405 with Ala or Asn in rat APB caused a change of substrate specificity toward fluorogenic substrates [12].

Residues responsible for substrate specificity of M1 aminopeptidases have been identified. Here, Gln181 of human endoplasmic reticulum aminopeptidase 1 (ERAP1) was found to be a residue important for ERAP1 substrate specificity [13]. Analysis of the recently resolved ERAP1 crystal structure revealed that Gln181 of human ERAP1 occupies the S1 site of the enzyme [14,15]. Replacement of Gln181 with Asp demonstrated that the mutant enzyme has a preference for basic amino acids, suggesting that Asp in this position is important for basic amino acid preference. Moreover, ERAP2 shows a preference for basic amino acids and has an Asp residue in the corresponding site and substitution of this residue with Gln abrogated the basic amino acid preference of this enzyme [13,16]. However, the corresponding residue of human APB is a Gln, despite the observation that this enzyme has a preference for basic amino acids.

Site-directed mutagenesis is a useful approach to elucidate mechanisms of enzymatic activity [17–19]. Using this approach, two consensus motifs, HEXXH$(X)_{18}$E and GXMEN, were identified to be critical for the hydrolytic activities shared by the M1 family of aminopeptidases. Both His residues and the second Glu in the HEXXH$(X)_{18}$E motif function as zinc ligands and are essential for the catalytic activity of M1 aminopeptidases, whereas the first Glu residue polarizes a water molecule and promotes nucleophilic attack of the carbonyl carbon of the peptide bond, forming a tetrahedral intermediate. On the other hand, Glu and Asn in the GXMEN motif are important for exopeptidase specificity through interaction with the N-terminal amide of substrates [17,20,21]. Moreover, it was recently reported that the Gly in GXMEN contributed to enzymatic activity and substrate specificity of M1 aminopeptidases [22,23].

In this study, we have analyzed the role of human APB Gln169, which corresponds to Gln181 of ERAP1, by site-directed mutagenesis analysis. We found that Gln169 was required for the preferential cleavage of Arg- and Lys-MCA fluorogenic substrates, chloride anion sensitivity and the maximal enzymatic activity toward several peptide substrates with N-terminal basic amino acids. Our data suggest that Gln169 (together with Asp406) is important to maintain the basic amino acid preference of APB, which is essential for its function as a precursor processing enzyme of hormones and neurotransmitters.

2. Materials and methods

2.1. Molecular modeling of the human APB

The recently published X-ray crystallographic structure of the human LTA4H with the RSR substrate (PDB ID: 3B7S) was used as a template for modeling the catalytic site of the human APB using the SWISS-MODEL Internet server (http://swissmodel.expasy.org/). To optimize the structure, hydrogen atoms were added to the initial model and the protonation states were assigned using the Protonate-3D tool within the MOE2012.10 software package (Chemical Computing Group, Montreal, QC, Canada). Energy minimization was then carried out using the AMBER12:EHT forcefield. For docking calculations, the backbones of the model (APB and RSR) were fixed and side-chain conformations were then subjected to another refinement procedure, including several rounds of energy minimization (until convergence) using a Molecular Mechanics method. We also built models of Gln169Asn and Gln169Glu, as well as the wild-type. The structures were represented using the CueMol program (R. Ishitani, CueMol: Molecular Visualization Framework).

2.2. Site-directed mutagenesis

DNA sequences encoding mutant APBs were generated by the polymerase chain reaction (PCR) using the full-length human APB cDNA (RIKEN clone ID: IRAL002L13) as the template. PCRs were carried out in 0.2 mL microcentrifuge tubes with 30 μL reaction volumes and performed for 1 cycle at 95 °C for 4 min, followed by 20 cycles at 95 °C for 1 min, at 55 °C for 1 min, and at 68 °C for 18 min using Pyrobest DNA polymerase (Takara, Otsu, Japan). The sense primer A (5′-TAGAGGATCCATGGCGAGCGGCGAGCATT-3′), containing a *Bam*HI sequence for directional cloning and the initiation ATG codon (underlined), and antisense primers complementary to the desired sequences were used for the amplification of upstream fragments. Downstream fragments were amplified using mutagenic sense primers and primer B (5′-TGAGTCGACCTAACTGCCCTTGGGT-3′) containing the *Sal*I sequence.

The two products of these reactions were used as templates for the second round of PCR. Secondary PCR was carried out with primers A and B for 1 cycle at 95 °C for 4 min, followed by 20 cycles at 95 °C for 1 min, at 55 °C for 1 min, and at 68 °C for 18 min. The resultant products were inserted into the expression vector, pQE-30 vector (Qiagen, Valencia, CA, USA), using the TOPO-cloning system (Invitrogen, La Jolla, CA). The DNA sequences of the products were commercially confirmed (Operon Biotechnologies, Tokyo, Japan).

2.3. Expression and purification of the recombinant APB and the mutants

The resultant plasmids were transformed into the *Escherichia coli* JM109 strain to produce recombinant proteins. For the expression of the recombinant human APB or its mutants, bacteria were precultured until the OD_{600} reached 0.5–0.7. To induce synthesis of the recombinant proteins, isopropyl β-D-thiogalactopyranoside (IPTG) was added to the cultures to a final concentration of 0.2 mM. Cells were cultured overnight at 28 °C in 1 L of Luria–Bertani broth containing 100 μg/mL ampicillin.

To purify the recombinant proteins, bacteria were collected by centrifugation at 4000 ×*g* for 20 min and sonicated on ice five times for 30 s using a sonicator (Model 250D sonifier, Branson, Danbury, CT, USA). Soluble proteins were recovered after centrifugation at 10,000 ×*g*, and the supernatants were applied to a DEAE Toyopearl (Tosoh, Tokyo, Japan) column equilibrated in 25 mM Tris/HCl buffer (pH 7.5) and eluted with 25 mM Tris/HCl buffer (pH 7.5) containing 50 mM NaCl. The eluates were applied to a TALON (Clontech, Palo Alto, CA, USA) column and eluted with 150 mM imidazole. The active fractions were collected, concentrated and subjected to further characterization.

2.4. Measurement of APB aminopeptidase activity

The aminopeptidase activity of the recombinant human APB and its mutants was determined using fluorogenic substrates, aminoacyl-4-metheylcoumaryl-7-amides (aminoacyl-MCAs). The reaction mixtures contained various concentrations of amino-acyl-MCAs and the enzyme (0.1 μg/mL) in 50 μL of 20 mM Tris/HCl buffer (pH 7.4) with 150 mM NaCl, and were incubated at 37 °C for 15 min. The amount of released 7-amino-4-methylcoumarin was measured by spectrofluorophotometry (MTP-810Lab, Hitachi, Tokyo, Japan) at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The kinetic parameters were calculated from Lineweaver–Burk plots. The results are represented as K_m, k_{cat} and k_{cat}/K_m values. All measurements were performed in triplicate.

2.5. Effect of sodium chloride and inhibitors on APB aminopeptidase activity

To measure the effect of sodium chloride on aminopeptidase activity, the recombinant proteins (0.1 μg/mL) and 25 μM Arg-MCA were mixed