Biochimie 93 (2011) 730–741

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Mutation in the substrate-binding site of aminopeptidase B confers new enzymatic properties

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ARTICLE INFO

Article history:
Received 3 November 2010
Accepted 24 December 2010
Available online 13 January 2011

Keywords:
Zn^{2+}-metallopeptidase
Miniglucagon
Hormone processing
Neuropeptide processing
Leukotriene A_4 hydrolase
M1 family

ABSTRACT

Aminopeptidase B (Ap-B) catalyzes the cleavage of arginine and lysine residues at the N-terminus of various peptide substrates. *In vivo*, it participates notably in the miniglucagon and cholecystokinin 8 processing, but the complete range of physiological functions of Ap-B remains to be discovered. Ap-B is a member of the M1 family of Zn^{2+}-metallopeptidases that are characterized by two highly conserved motives, GXMEN (potential substrate binding site) and HEXXHX^{18}E (Zn^{2+}-binding site). In this study, mutagenesis and molecular modelling were used to investigate the enzymatic mechanism of Ap-B. Nineteen rat Ap-B mutants of the $G_{298}XM_{300}E_{301}N_{302}$ motif and one mutant of the $HEIS_{328}HX^{18}E$ motif were expressed in *Escherichia coli*. All mutations except $G_{298}P$, $G_{298}S$, and $S_{328}A$ abolished the aminopeptidase activity. The $S_{328}A$ mutant mimics the sequence of bovine Ap-B Zn^{2+}-binding site, which differs from those of other mammalian Ap-B. This mutant conserved a canonical Ap-B activity. $G_{298}S$ and $G_{298}P$ mutants exhibit new enzymatic properties such as changes in their profile of inhibition and their sensitivity to Cl^- anions. Moreover, the $G_{298}P$ mutant exhibits new substrate specificity. A structural analysis using circular dichroism, fluorescence spectroscopy, molecular modelling and dynamics was performed to investigate the role that residue G_{298} plays in the catalytic mechanism of Ap-B. Our results show that G_{298} is essential to Ap-B activity and participates to the substrate specificity of the enzyme.

1. Introduction

NRD convertase [1] and Cathepsin L [2] cleave prohormones on the NH_2-terminus side of basic amino acid doublets. Aminopeptidase B (Ap-B) hydrolyses Arg or Lys residue at the NH_2-terminus of various peptides [3]. Working together, these peptidases process somatostatin-28 into somatostatin-14 *in vitro* [4,5], glucagon into miniglucagon in the α-cells of the islets of Langerhans [6,7], enkephalins in various tissues [8], cholecystokinin in mouse brain [9] and POMC in the pituitary [10].

Ap-B is a secreted enzyme ubiquitously expressed in mammals and whose expression level is both tissue- and species-dependent [3,11–16]. *In vitro*, Ap-B has also a residual capacity to hydrolyze leukotriene A_4 (LTA_4) into the pro-inflammatory lipid mediator leukotriene B_4 (LTB_4) [12]. The bi-functional nature of Ap-B is supported by a close structural relationship with LTA_4 hydrolase (LTA_4H; EC 3.3.2.6; 33% identity, 48% similarity) [12], which hydrolyses LTA_4 into LTB_4 *in vivo*, and

Abbreviations: ACE, angiotensine converting enzyme; Ap-A, glutamyl aminopeptidase; Ap-B, aminopeptidase B; Ap-N, aminopeptidase N; Ap-O, aminopeptidase O; Ap-Q, aminopeptidase Q; Bac-rAp-B, recombinant His-tagged rat aminopeptidase B produced from baculovirus infected insect cells; CCK, cholecystokinin; ERAP1, endoplasmic reticulum aminopeptidase 1; ERAP2, endoplasmic reticulum aminopeptidase 2; His-rAp-B, recombinant His-tagged rat aminopeptidase B produced from *E. coli*; IRAP, insulin regulated membrane aminopeptidase; L-aa β-NA, L-amino acid β-naphthylamide; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; LTA_4H, leukotriene A_4 hydrolase; PSA, puromycin sensitive aminopeptidase; rAp-B, rat aminopeptidase B; tACE, testis angiotensine converting enzyme; TRH-DE, thyrotropin-releasing hormone degrading enzyme.

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doi:10.1016/j.biochi.2010.12.015

exhibits an aminopeptidase activity *in vitro* [17]. Both enzymes belong to the M1 family of Zn^{2+}-dependent metallopeptidases whose members are characterized by the presence of two conserved motives in their primary structures, GXMEN and $HEXXHX^{18}E$ [12,15,17–21]. A 3D model of Ap-B was constructed [22] based on the crystal structure of human LTA_4H in complex with zinc ion and bestatin [23].

The catalytic mechanism of the enzymes belonging to the M1 family was originally deduced from that of thermolysine (M4 family of Zn^{2+}-endoproteases) [24] and was recently detailed for LTA_4H [25]. The $HEXXHX^{18}E$ motif is involved in the binding of the Zn^{2+} cation. In the absence of substrate, the zinc atom is tetracoordinated by the histidine residues, the second glutamate residue of this pattern, and a water molecule. Once the substrate is bound in the active site, it displaces the zinc-associated water molecule and chelates the zinc ion by its free amine and carbonyl oxygen [25]. The first glutamate of the pattern participates to the catalysis reaction, probably acting as a general base in the nucleophilic attack of the carbonyl group of the peptide bond, and as an acid catalyst to give a proton to the leaving amine moiety [25]. Although Ap-B and LTA_4H from Mammals show a highly conserved sequence of the Zn^{2+}-binding motif (HEISH), bovine Ap-B [26] exhibits a different sequence (HEIAH), which is found in several other aminopeptidases of the M1 family, e.g. the putative LTA_4H from *Drosophila melanogaster* (accession number Q7KT44), *Anopheles gambiae* (accession number Q7Q192), *Caenorhabditis elegans* (accession number O44183), and *C. briggsae* (accession number Q61MW9). Interestingly, bovine Ap-B shows a different substrate specificity compared to the rat enzyme, since it is able to preferentially cleave the Arg-methyl-coumarin (MCA) substrate, but also to a lesser extend: Asn-, Leu-, Met-, Asp-, Ser- and Lys-MCA substrates [26].

The GXMEN motif was proposed to participate to the transition state stabilization and to the aminopeptidase specificity. The glutamate and asparagine residues seem to be implicated in the binding of the NH_2-terminus of substrates [25,27–32]. Co-crystallisation of the LTA_4H-$E_{296}Q$ mutant with a tripeptide showed that hydrogen bonds are established between the carbonyl oxygen of the leaving P′1 residue of the substrate and the amide nitrogen of the glycine residue [25]. Analysis of mutations of this glycine residue in the GXMEN motif is difficult, since the function of this amino acid depends on the substrate specificity of the aminopeptidase and the rearrangement of the active site during the catalysis process.

Herein, 19 rat Ap-B (rAp-B) mutants of the $G_{298}XM_{300}E_{301}N_{302}$ motif were constructed and expressed in *Escherichia coli*. Among the 19 Ap-B mutants, only two mutations led to an active enzyme, $G_{298}P$ and $G_{298}S$. The $G_{298}S$ mutant conserves the Arg and Lys substrate specificity of the wild-type enzyme, whereas the $G_{298}P$ mutant gains a new activity against Ala and Pro residues at the N-terminus of L-amino acid β-naphthylamide (L-aa β-NA), together with a modified inhibition profile and a loss of Cl^- anion sensitivity. Analysis of the mutants using circular dichroism shows that $G_{298}P$ and $G_{298}S$ exhibit only small variations of secondary structures compared to rAp-B. Fluorescence spectroscopy on $G_{298}P$ and $G_{298}S$ mutants compare to the wild type protein does not reveal noteworthy variation. Molecular modelling and dynamics were also used to study these new enzymes and the role of the G_{298} residue in Ap-B.

Moreover, the $HEIA_{328}H$ mutant was constructed, expressed, purified to homogeneity, and characterized for its catalytic specificity, in order to analyze the effect of a bovine-like mutation in the HEISH motif of rAp-B. The results show that the $S_{328}A$ mutant and the recombinant rAp-B exhibit similar properties. This demonstrates that the alanine of this motif is not directly implicated in the substrate specificity of bovine Ap-B.

2. Materials and methods

2.1. Site-directed mutagenesis

The pIVEX2.4-Ap-B recombinant expression vector was used for site-directed mutagenesis [22] and mutants were generated with the QuickChange® Multi Site-Directed Mutagenesis kit according to the manufacturer specifications (Stratagene Europe, Amsterdam, Netherlands). This system allows randomizing (X) the targeted amino acid residues using oligonucleotides containing degenerate codons (site-specific saturation mutagenesis; see below). All the primers used for mutagenesis were 5′-phosphorylated. A single oligonucleotide per site was used in each experiment. The mutagenic codon was underlined in the oligonucleotide sequence. The targeted amino acids and their corresponding mutagenic primers were the followings: $G_{298}X$, 5′-CCATCTTTCCCGTTTNNNGGAATGGAGAATCCC-3′; $G_{298}S$, 5′-CCATCTTTCCCGTTTAGTGGAATGGAGAATCCC-3′; $G_{298}A$, 5′-CCATCTTTCCCGTTTGCGGGAATGGAGAATCCC-3′; $G_{298}P$, 5′-CCATCTTTCCCGTTTCCGGGAATGGAGAATCCC-3′; $M_{300}X$, 5′-CCCGTTTGGAGGANNNGAGAATCCCTGCCTG-3′; $E_{301}X$, 5′-CGTTTGGAGGAATGNNNAATCCCTGCCTGACC-3′; $N_{302}X$, 5′-TTTGGAGGAATGGAGNNNCCCTGCCTGACCTTT-3′; $S_{328}A$, 5′-TGTGCGCGATCTCGTGGATGATGACGTCGG-3′. Generated mutants were identified by direct sequencing on both strands using the dideoxy chain-termination procedure (Genome Express facilities, Meylan, France). The $G_{298}G$ conservative mutant presented in Table 1 constitute a positive control in the site-directed mutagenesis experiments. Other putative conservative mutants such as $M_{300}M$, $E_{301}E$ and $N_{302}N$ could also be considered as controls although their corresponding codon remains unchanged. However, it cannot be preclude that they come from the undigested pIVEX2.4-Ap-B parental DNA template. Some mutants, such as $G_{298}A/P/S$, were obtained by site-specific saturation mutagenesis and then by classical site-directed mutagenesis. The $S_{328}A$ mutant was constructed only by classical site-directed mutagenesis.

2.2. Production of rAp-B in E. coli

The pIVEX2.4-Ap-B recombinant plasmid was used to produce wild-type (His-rAp-B) and mutated recombinant rAp-B (NH_2-terminal His-tagged proteins) with a T7 promoter-driven system and a BLi5 *E. coli* strain as described in [22]. Briefly, 1 mL of LB medium supplemented with 20 μg/mL chloramphenicol and 100 μg/mL ampicillin were inoculated with Bli5 cells harbouring the pIVEX2.4-Ap-B plasmid and incubated overnight at 37 °C with agitation. The overnight culture was then diluted to 1:50 with 50 mL of fresh LB medium containing 100 μg/mL ampicillin and was grown under vigorous shaking at 37 °C until the OD_{600} reached 0.6. Isopropyl β-D-1-thiogalactoside (Sigma–Aldrich, Saint Quentin Fallavier, France) was added to a final concentration of 1 mM and the expression culture was grown at 37 °C under agitation for 2 hours. Cells were then harvested by centrifugation at 4000 × g for 5 min and stored at −80 °C until use or processed immediately as described below for western blotting, purification and enzymatic activity assays.

2.3. Production of rAp-B in baculovirus-infected cells

H5 cells were grown in liquid cultures (1 liter flask) at 28 °C in X-Press medium (BioWhittaker, France) supplied with fungizone (2.5 mg/mL; BioWhittaker, France) and gentamycine (50 mg/mL; BioWhittaker, France). Cells were infected at a density of 2×10^8 cells/liter with 1 mL of rAp-B-HIS-BAC virus stock expressing His-tagged rAp-B [33]. After 3 days of infection, cells were harvested by centrifugation 10 min at 1500 × *g* and the culture medium was collected. The recovered infected cell culture medium supernatant (500 mL) was concentrated to 200 mL and equilibrated