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The M1 family of vertebrate aminopeptidases: Role of evolutionarily conserved tyrosines in the enzymatic mechanism of aminopeptidase B

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A B S T R A C T

Aminopeptidase B (Ap-B), a member of the M1 family of Zn²⁺-aminopeptidases, removes basic residues at the NH₂-terminus of peptides and is involved in the *in vivo* proteolytic processing of miniglucagon and cholecystokinin-8. M1 enzymes hydrolyze numerous different peptides and are implicated in many physiological functions. As these enzymes have similar catalytic mechanisms, their respective substrate specificity and/or catalytic efficiency must be based on subtle structural differences at or near the catalytic site. This leads to the hypothesis that each primary structure contains a consensus structural template, strictly necessary for aminopeptidase activity, and a specific amino acid environment localized in or outside the catalytic pocket that finely tunes the substrate specificity and catalytic efficiency of each enzyme. A multiple sequence alignment of M1 peptidases from vertebrates allowed to identify conserved tyrosine amino acids, which are members of this catalytic backbone. In the present work, site-directed mutagenesis and 3D molecular modeling of Ap-B were used to specify the role of four fully (Y₂₈₁, Y₂₂₉, Y₄₁₄, and Y₄₄₁) and one partially (Y₄₀₉) conserved residues. Tyrosine to phenylalanine mutations allowed confirming the influence of the hydroxyl groups on the enzyme activity. These groups are implicated in the reaction mechanism (Y₄₁₄), in substrate specificity and/or catalytic efficiency (Y₄₀₉), in stabilization of essential amino acids of the active site (Y₂₂₉, Y₄₀₉) and potentially in the maintenance of its structural integrity (Y₂₈₁, Y₄₄₁). The importance of hydrogen bonds is verified by the Y₂₂₉H substitution, which preserves the enzyme activity. These data provide new insights into the catalytic mechanism of Ap-B in the M1 family of aminopeptidases.

Keywords:

Aminopeptidases
Zn²⁺-metallopeptidase
Leukotriene A₄ hydrolase
M1 family
Tyrosine

1. Introduction

Aminopeptidase B (Ap-B) is a Zn²⁺-aminopeptidase that specifically hydrolyzes the peptide bond on the carbonyl side of arginine and/or lysine residues present at the NH₂-terminus of peptides [1]. Ap-B is ubiquitously expressed in vertebrates [2–4] and participates, in synergy with endoproteases, in the maturation of hormones and neuropeptides. To date, only two of its physiological substrates are identified: NRD convertase hydrolyses the glucagon into Arg-Arg-miniglucagon that is converted by Ap-B into miniglucagon, a peptide involved in the maintenance of glucose homeostasis [5]; cathepsin L hydrolyzes cholecystokinin (CCK) precursors into CCK9, which is processed by Ap-B into CCK8, a pleiotropic neuropeptide [6]. This enzyme may also be implicated in the *in vivo* processing of enkephalins [7] and Arg-extended forms of human insulin [8]. More recently, Ap-B was identified as a new

Abbreviations: Ap-A, aminopeptidase A; Ap-B, aminopeptidase B; Ap-N, aminopeptidase N; Ap-O, aminopeptidase O; Ap-Q, aminopeptidase Q; RNPEP-L1, arginyl aminopeptidase-like 1; CCK, cholecystokinin; ERAP, endoplasmic reticulum aminopeptidase; His-rAp-B, His-tagged rat Ap-B; IRAP, insulin-responsive aminopeptidase; L-amino acid β-NA, L-amino acid β-naphthylamide; LTA4, leukotriene A₄; LTA4H, leukotriene A₄ hydrolase; LTB₄, leukotriene B₄; PSA, puromycin-sensitive aminopeptidase; TRH-DE, thyrotropin-releasing hormone degrading enzyme.

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cytoplasmic degrading enzyme, interfering with the maturation of a major CD8⁺ T cell epitope of the human cytomegalovirus phosphoprotein 65 [9].

Molecular cloning of rat and human Ap-B [3,10] identified this protein as a member of the M1 family of aminopeptidases, belonging to the MA clan of metallopeptidases (Merops, the peptidase database; <http://merops.sanger.ac.uk/>). This clan, also called zincins clan [11], is divided into 39 families of endo- or exopeptidases that require a Zn²⁺ cation for their activity. These enzymes are unified by the presence in their primary structure of a HEXXH motif, in which histidine residues are Zn²⁺ ligands and glutamate has a catalytic activity. More specifically, the M1 family of aminopeptidases belongs to the MA(E) subclan, also named gluzincins clan [11] that is defined by the presence of a second glutamate, located 18 residues downstream the HEXXH motif, and acting as a third Zn²⁺ ligand. A tyrosine residue located 80 to 90 residues downstream the HEXXH motif completes this M1 signature. The catalytic role of this residue, together with the Zn²⁺ cation, is to polarize the carbonyl of the hydrolyzed peptide bond [12]. This consensus pattern, HEXXH¹⁸EX^{80–90}Y, allows to classify about 2900 protein sequences in the M1 family (Merops; <http://merops.sanger.ac.uk/>). About 77% of these sequences contain the motif (G/A/H/V) (G/A)MEN. This second pattern is usually located 22 to 38 residues upstream of the HEXXH motif and is involved in the recognition and binding of the substrate. The glutamic acid plays a key role in positioning the α amino group of the peptide substrate while the other residues participate in the formation of the S1 pocket [12–17].

M1 aminopeptidases are largely distributed among the living world and about 55 different enzymes were predicted in Archaea, Bacteria and Eukaryotes (Plants to Vertebrates). However, most of these putative enzymes have not been characterized and no information on their cleavage specificity and enzymatic mechanism is available. In contrast, the M1 aminopeptidases expressed in mammals, 12 different enzymes, are much better characterized [Aminopeptidase A (Ap-A), B (Ap-B), N (Ap-N), O (Ap-O), Q (Ap-Q); arginyl aminopeptidase-like 1 (RNPEP-L1); Endoplasmic Reticulum Aminopeptidase 1 and 2 (ERAP1, ERAP2); Insulin-Responsive Aminopeptidase (IRAP); Leukotriene A₄ hydrolase (LTA₄H); Puromycin-Sensitive Aminopeptidase (PSA); Thyrotropin-Releasing Hormone Degrading Enzyme (TRH-DE)].

The crystal structure of the human LTA₄H was the first 3D structure determined in the M1 family [18]. More recently, several other structures were obtained: Ap-N from *Escherichia coli* [19], *Neisseria meningitidis* [20], *Plasmodium falciparum* [21], porcine [17]

and human [22]; human ERAP1 [23]; human ERAP2 [24]. The main difference between these structures is the presence of three (LTA₄H) or four (Ap-N, ERAP1 and 2) structural domains, three of them appearing similar in all proteins (Fig. 1). Consequently, these enzymes can be classified into two subfamilies according to their sizes and structural homology. The first subfamily consists of Ap-A, Ap-N, Ap-Q, ERAP1, ERAP2, IRAP, PSA and TRH-DE. The second family is composed of Ap-B, Ap-O, LTA₄H and RNPEP-L1. In this subfamily, LTA₄H is the best characterized. LTA₄H is bifunctional *in vivo* with an epoxide hydrolase activity that converts, in the arachidonic acid pathway, the LTA₄ into the inflammatory mediator leukotriene B₄ (LTB₄). The enzyme also exhibits an aminopeptidase activity with a broad specificity being able to hydrolyze substrates such as Leu, Ala or Pro-4-nitroanilide [25]. Recently, a neutrophil chemoattractant, Pro-Gly-Pro (PGP), was found to be one of the physiological substrates of the human LTA₄H [26]. Paradoxically, degradation of this peptide by the enzyme facilitates the resolution of inflammation [27].

Ap-B is closely related to LTA₄H (37% sequence identity, 45% similarity). Moreover, Ap-B is bifunctional *in vitro*, with an aminopeptidase and a residual LTA₄ hydrolase activity [10]. Since there is no Ap-B structural data available, a 3D model was built based on the crystallographic structure of LTA₄H [28]. Additionally, the co-crystallization of the LTA₄H^(E296Q) mutant with the R-A-R tripeptide substrate (pdb: 3B7T; 12) revealed important structural information that have allowed a better understanding of the enzymatic mechanism of Ap-B after docking of R-A-R ligand in the active site [16].

The important conserved residues for all the vertebrate peptidases of the M1 family were identified using multiple-sequence alignments. We focused our attention on conserved tyrosine residues. The roles of these amino acids in protein structures and biological functions are associated to their involvement in polar hydrogen- π interactions (Hp- π) due to the hydroxyl group and to the aromatic ring, which are the Hp- π donor and acceptor, respectively [29]. The Hp- π interactions are specific to three amino acids (H, W, Y) and may be responsible for some of the flexibility and dynamics of proteins.

Four fully conserved Tyr residues were identified, corresponding to Y₂₂₉, Y₂₈₁, Y₄₁₄ and Y₄₄₁ in Ap-B primary structure. Y₄₀₉, a partially conserved residue, was also studied. Y₂₂₉ is located in the β -sheet domain (54–237) of Ap-B (Fig. 1A). Y₂₈₁, Y₄₀₉, Y₄₁₄ and Y₄₄₁ are in the central catalytic domain (238–489), which contains the G₂₉₈GMEN and H₃₂₅EXXH(X¹⁸)E motifs. More precisely, Y₂₈₁ is located on a loop, and points to a β sheet that contains the GAMEN

Fig. 1. Schematic representation showing position of the five tyrosine residues (Y₂₂₉, Y₂₈₁, Y₄₀₉, Y₄₁₄ and Y₄₄₁) in the 3D structural model of Ap-B (A) and the corresponding residues in human Ap-N (pdb: 4FYQ) (B). The domain assignment of the crystallized LTA₄H (pdb: 3b7t; SCOP, <http://scop.mrc-lmb.cam.ac.uk/scop/index.html>) was used to define the localization of residues in Ap-B 3D model. Three structural domains are defined for Ap-B: NH₂-terminus (54–237, yellow), central (238–489, gray), COOH-terminal (490–650, orange). Four domains are identified in Ap-N 3D structure (pdb: 4FYQ): NH₂-terminus (64–286, yellow), central (287–549, gray), hinge (550–635, green), COOH-terminal (636–967, orange).

motif. Y₂₂₉ is on a small α helix between two β strands, and head to tail with Y₄₁₄. The latter is positioned on a α helix and oriented to the central catalytic cavity. Y₄₀₉ is on a loop at the extremity of a α helix and in proximity of Y₄₁₄. Finally, Y₄₄₁ belongs to a α helix exposed to the protein surface. In the human Ap-N structure, the positioning of the corresponding Y (or F) residues is similar, Y₂₇₈ is in the β -strand domain (64–286) and Y₃₃₆, F₄₇₂ (Y₄₀₉ in Ap-B), Y₄₇₇ and Y₅₀₃ are in the catalytic domain (287–549; Fig. 1B). In LTA₄H, Y₃₈₃ (Ap-B, Y₄₁₄; Ap-N, Y₄₇₇; Fig. 1) is located in the active site and its mutation leads to a selective abrogation of the aminopeptidase activity [30]. Y₃₈₃ was first proposed as a proton donor to the nitrogen of the hydrolyzed peptide bond in the catalytic mechanism [30]. So far, the proposed role of Y₃₈₃ is to participate in the polarization of the carbonyl bond, to increase the electropositive character of the carbon necessary for a nucleophile attack by a water molecule. This residue seems also to stabilize the intermediate oxyanion of the hydrated peptide [12]. As this tyrosine residue is conserved in the whole sequences of the M1 family, and appears to be clearly located in the active site of the crystallized aminopeptidases, such a function in the catalytic mechanism could be generalized. In the present report, mutagenesis experiments were realized to confirm the putative contribution of the Ap-B corresponding Y₄₁₄ residue.

Three other tyrosine residues Y₂₂₉, Y₂₈₁ and Y₄₄₁ are fully conserved in the M1 family. Despite the fact that they are distant from the active site, Y to F mutations were performed to determine the importance of their hydroxyl group. In the LTA₄H of *Saccharomyces cerevisiae*, Y₂₄₄ (Y₂₂₉ in Ap-B) is essential to the aminopeptidase activity and seems to be involved in the stabilization of the transition state [31]. More recently, the co-crystallization of the LTA₄H with the RAR tripeptide substrate allowed attributing a more precise and essential function for this residue, a function that could be generalized to all the vertebrate members of the M1 family. In the case of the Ap-B, our results suggest that the hydroxyl group of Y₂₂₉ is crucial for an indirect stabilization and polarization of the substrate in the Michaelis complex. The hydroxyl group of Y₂₂₉ establishes a hydrogen bond with the carboxylate of E₃₀₀ in the GAMEN motif, leading to stabilizing this crucial amino acid implicated in the anchorage of the NH₂-terminus of the substrate. It can be replaced by a histidine, which is able to play similar functions [29]. In contrast, the hydroxyl groups of Y₂₈₁ and Y₄₄₁ of the Ap-B are not involved into a direct interaction with the defined residues of first shell of the active site, but rather intervene in a secondary structural stabilization, important for the enzymatic molecular dynamic.

We also focused our attention on Y₄₀₉, which is replaced by a phenylalanine residue in some vertebrate LTA₄H sequences (e.g. *Xenopus laevis*) and in aminopeptidases with four structural domains and Ap-O. The Y₄₀₉F mutation shows the importance of the hydroxyl group of Y₄₀₉, an amino acid that probably potentiates the catalytic role of Y₄₁₄ in the enzymatic mechanism.

This work emphasizes that amino acids of the second shell, as well as more distant residues, are essential for the chemical properties of amino acids of the first shell of the active site. They are also important in the induced fit of the substrate–enzyme complex and in the catalysis. Moreover, this analysis could highlight similarities and differences in the enzymatic mechanisms of the aminopeptidases belonging to the M1 family, which could facilitate the conception of specific inhibitors.

2. Materials and methods

2.1. Multiple sequence alignments

The BLASTP program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used, with default parameters (BLOSUM62 matrix), to identify

members of the M1 family of metalloexopeptidases. The non-redundant protein sequences database (nr; update date 2013/09), restricted to vertebrate species (taxid: 7742), was queried with the human Ap-B protein sequence [3] and 990 hits exhibiting an E value between 0 and 1×10^{-8} were observed. These 990 protein sequences were aligned using CLUSTALW [<http://www.ebi.ac.uk/clustalw/>]. The incomplete sequences, and/or sequences that exhibited obvious mistranslations resulting from sequencing errors, were deleted from the alignment. Among these proteins, a unique sequence per species was extracted, after checking the quality of the sequence with multiple alignments between sequences of the same aminopeptidase within the same species. Then the alignment was refined manually to minimize the number of gaps. Finally, 273 vertebrate protein sequences were aligned to check the full conservation of both signatures of the M1 family [HEXXHX¹⁸EX^{80–90}Y; (G/A/H/V) (G/A)MEN], and to identify the fully conserved tyrosine residues. In order to provide a sequence alignment of reasonable size, only the human proteins are shown in Fig. 2.

2.2. Site-directed mutagenesis of tyrosine residues

The pIVEX2.4-Ap-B recombinant expression vector was used for site-directed mutagenesis [28] and mutants were generated with the QuickChange[®] Multi Site-Directed Mutagenesis kit according to the manufacturer specifications (Stratagene Europe, Netherlands). A single 5' phosphorylated oligonucleotide per site was used in each experiment. The targeted amino acids and their corresponding mutagenic primers were the followings:

5'-CCAAAGCTATCAGNNNGGAGGGGATGGGC-3' for Y₂₂₉
 5'-CCAAAGCTATCAGAAAGGAGGGGATGGGC-3' for Y₂₂₉F
 5'-AGCCTTTTCGGACCTTTTGTGGGAAGA-3' for Y₂₈₁F
 5'-GACCCAGATGACACCTTTAATGAAACCCCTAC-3' for Y₄₀₉F
 5'-TACAATGAAACCCCTTTGAGAAAGTTACTGC-3' for Y₄₁₄F
 5'-TACAATGAAACCCCNNGGAGAAAGTTACTGC-3' for Y₄₁₄
 5'-AAGTTTCTCAAGGCCTTTGTAGATGAGTTTAAG-3' for Y₄₄₁F

The mutagenic codon is underlined in the oligonucleotide sequence. Several mutants (Y₂₂₉, Y₄₁₄) were obtained using one oligonucleotide containing a degenerate codon (NNN) in order to generate mutant collection. The nucleotide sequence of each mutant was determined on both strands [Cogenics, United Kingdom].

2.3. Production of His-tagged Ap-B in *E. coli*

The pIVEX2.4-Ap-B recombinant plasmid was used to produce wild-type His-tagged rat Ap-B (His-rAp-B) and mutated His-rAp-B with a T7 promoter-driven system and a Bli5 *E. coli* strain as described previously [28]. Briefly, 800 μ L of SOB medium with 20% glucose were inoculated with Bli5 cells harboring the pIVEX2.4-Ap-B plasmid or mutants and incubated 1 h at 37 °C under agitation, then supplemented with 25 μ g mL⁻¹ chloramphenicol and 100 μ g mL⁻¹ ampicillin and incubated overnight at 37 °C under agitation. The overnight culture was diluted to 1:50 with 50 mL of fresh LB medium containing 100 μ g mL⁻¹ ampicillin and was grown under vigorous shaking at 37 °C until the OD₆₀₀ reached 0.6. Isopropyl β -D-1-thiogalactoside (Sigma–Aldrich, France) was added to a final concentration of 1 mM and the expression culture was grown overnight at 25 °C under agitation. Cells were harvested by centrifugation at 4500 $\times$ g for 20 min and stored at –20 °C. For a preliminary screening of the activity of the mutant compared to the wild type enzyme, 200 μ L of competent Bli5 cells were transformed with 300 ng of plasmid. Then the

ERAP1_944aa	STYLVAFIISDFESVSKMTKSGVKVSVYAVPDKI--NQADYALDAAVTLLEFYEDYFSIPYPLPKQDLAAIP-DFQSGAMEN	321
ERAP2_960aa	STYLVAIVCDFHVSQTTSSGKVSIVASPDKW--NQTHYALQASLKLDFYKDYFDINYPPLPKDLIAIP-DFASGAMEN	338
IRAP_1026aa	STYLVAFIVGEMKNLSQDVN-GTLVSIYAVPEKI--GQVHHALETTVKLLFEYQNYFEIQYPLKKLDLVAIP-DFEAGAMEN	432
Ap-A_959aa	STYLVCFVAVHQFDSVERISNSGKPLTIYVQPEQK--HTAETAAANITKSVFDYFEEYFAMNYSPLPKLDKIAIP-DFGTGAMEN	361
PSA_921aa	STYLVAFVVEGYDFVETRSKDGVCVRVYTFVVGKA--EQGKFALEVAAKTLPFYKDYFNVPYPLPKIDLIAIA-DFAGAMEN	320
ApN_972aa	STYLLAFIVSEFTYVEKQAPNGVLIRIWARPSAIAEGHGYALNVTGPILNFFAGHYNTPYPLPKSDQIALP-DFNAGAMEN	356
ApQ_991aa	PTYLVAFVICDYDHVNRTER-GKEIRIWARPKDAIANGSADFALNITGPIFSFLEDLFNISYSLPKTDIIALP-SFDNHAMEN	383
TRH DE 1066aa	STYLLAWAICNFTYRETTTSGVVRVLYARPDAIRRGSGDYALHITKRLIEFYEDYFKVPYSLPKLDLLAVP-KHPYAAAMEN	463
LTA4H_611aa	PCYLIALVVGALSRQIGPRT----LVWSEKEQV--EKSAYEFS-ETESMLKIAEDLGPGYVWGQYDLLVLPSPFPYGGMEN	272
RNPEP_L1_729aa	PAYLVALVAGDLKPADIGPRS----RVWAEPCLL--PTATSKLSGAVEQWLSAAERLYGPMWGRYDIVFLPSPFPYVAMEN	330
Ap-B_650aa	PSYLIALAIGDLVSAEVGPRS----RVWAEPCLL--DAAKEEYNGVIEEFLATGEKLFPGYVWGRYDLLFMPPSPFPYGGMEN	302
	(229)	(281)
ERAP1_944aa	WGLTTYRESALLFDAEKSSASSKLGITMTVAHELAHQWFGNLVTMEWWDNLWLNEGFQAKFMEFVSVSVTHPELKVEDY-FFGKCF	
ERAP2_960aa	WGLITYRETSLLFDPKTSSASDKLWTVKIVIAHELAHQWFGNLVTMEWWDNLWLNEGFQAKYMELIAVNATYPELQFDDY-FLNVCF	
IRAP_1026aa	WGLLTFREETLLYDNTSSVADRKLVTKIIAHELAHQWFGNLVTMQWWDNLWLNEGFATFMEYFSLEKIFKELSSYED-FLDARF	
Ap-A_959aa	WGLITYRETNLLYDPEESASSNQORVAAVVAHELVHQWFGNIVTMDWDDDLWLNEGFASFEEFLGVNHAEKDWQMRDQ-MLLEDV	
PSA_921aa	WGLVITYRETALLIDPKNSCSSSRQWVALVVGHELAHQWFGNLVTMEWWDNLWLNEGFASWIEYLVDHCFPEYDIWTQ-FVSADY	
ApN_972aa	WGLVITYRENALLFDPLSSSISNKERVTVIAHELAHQWFGNLVTLEWWDNLWLNEGFASYVEYLGAADYAEPTWNKDLIVLNDVY	
ApQ_991aa	WGLMIFDESGLLLEPKDQLEKTKLISYIVSHEIGHQWFGNLVTMNWNNIWLNEGFASYFEFVINYFKPLPRNEIFFSNILH	
TRH DE 1066aa	WGLSIFVEQRILLDPSVSSISYLLDVTMVIVHEICHQWFGDLVTPVWVEDVWLKEGFAHYFEFVGTDYLYPGWNMEKQRFLLTDVL	
LTA4H_611aa	PCLTFVTPPTLLAGD-----KSLSNVIAHEISHSWTGNLVTNKTWDHFWLNEGHTVYLERHICGRLFGEKFRHFHALGGWGE	
RNPEP_L1_729aa	PCLTFIISISILESD-----EFLVIDVIEHVAHSWFGNAVTNATWEEMWLSGLATYAQRRIITTETYGAAFTCLETAFRLLDA	
Ap-B_650aa	PCLTFVTPCLLAGD-----RSLADVIEHISHSWFGNLVTNANWGEFWLNEGFTMYAQRRIISTILFGAAYTCLAAATGRAL	
	(409) (414)	(441)
ERAP1_944aa	DAM-EVDALNSSHVPSTP---VENPAQIREMFDDVSIEKGACILNMLRDYLSADA-FKSGIVQYLQKYSYKNTKNEDLWNSMASI	
ERAP2_960aa	EVI-TKDSLNSSRPISKP---AETPTQIQEMFDEVSYNKGACILNMLKDFLGEEK-FQKGIQYLKKFSYRNAKNDLDWSSLSNS	
IRAP_1026aa	KTM-KKDSLNSSHPISSSS---VQSSEQIEEMFDSLSYFKGASLLMLKTYLSEDV-FQHAIVLYLHNHSYASIQSDDLWDSFNEV	
Ap-A_959aa	LPVQEDDSILMSSHPIVVT---VSTPAEITSVFDGISYSGKASILRMLLEDWITPEK-FQKGCQIYLEKYQFKNAKTSDFWAALEEA	
PSA_921aa	TRAQELDALDNSHPIEVS---VGHPSVEDEIFDAISYSGKASVIRMLHDYIGDKD-FKKGMMMLYTKFQQKNAATEDLWESLENA	
ApN_972aa	RV-MAVDALASSHPLSTPASEVNTPAQISELFDISYSGKASVLRMLSSFLTEDL-FKKGLASYLHTFAYQNTIYLDLWDHLQEA	
ApQ_991aa	NI-LREDHALVTRAVAMKVENFTKTSEINELFDLETYSKGASMARMLSSFLNEHL-FVSALKSYLKTFSYSNAEQDDLWRHFQMA	
TRH DE 1066aa	HEVMLLDGLASSHPVS---QEVLOATDIDRVFDWIAYKKGAALIRMLANFMGHSV-FORGLQDYLTIHKYGNAARNDLWNTLSEA	
LTA4H_611aa	LQNSIKTFGET-HPFTKLVDLTD-VDPDVAYSVPYEGKFALLFYLEQLLGGPEVFLGFLKAYVEKFSYKSITTDWKFDFLYSH	
RNPEP_L1_729aa	LHRQMKLLGED-SPVSKLQVKLEPGVNPShLMNLFYIEKGKCYFVYYLSQLCGDPQRFDDFLRAYVEKYKFTSVVAQDLDSFSLF	
Ap-B_650aa	LR-QHMDITGEEHPINKLRVKIEPGVDPDDTYNETPYEKGKCFVSYLAHLVGDQDQDFNFKAYVDEKFKQSILADDFLEFYLEY	
	(409) (414)	(441)

Fig. 2. Multiple alignment of the primary structures of the human proteins of the M1 family. The sequence accession numbers are in brackets: ERAP1 (Q9NZ08), ERAP2 (Q6P179), IRAP (Q9UIQ6), Ap-A (Q07075), PSA (P55786), Ap-N (P15144), Ap-Q (Q6Q4G3), TRH-DE (Q9UKU6), LTA4H (P09960), RNPEP-L1 (Q9HAU8), Ap-B (Q9H4A4). The alignment was restricted to the region containing the conserved tyrosine residues. The two consensus signatures HEXXH¹⁸E and (G/A/H/V) (G/A)MEN, which defined the M1 family, are shown in red. The conserved tyrosine residues are colored in green. The Y₄₀₉ residue of Ap-B and its homolog in LTA4H are shown. The horizontal line separates the proteins belonging to the two subfamilies of the M1 peptidases described in the introduction section.

protocol was identical to that described above except that the protein was expressed in a 2 ml final volume and the expression culture was grown 2 h at 37 °C under agitation. Cells were then harvested by centrifugation at 4500 × g for 5 min and the pellet was resuspended in 200 µl of BugBuster master mix (Novagen, Merck Chemicals, United Kingdom). The samples were incubated 10 min at room temperature under agitation and centrifuged 20 min at 10,000 × g. The supernatant was removed to perform the enzyme activity assays.

2.4. Purification of the recombinant His-tagged Ap-B and mutants

pIVEX2.4-Ap-B transformed cell pellets from an expression culture of 100 mL (see above) were resuspended in 10 mL of lysis buffer (0.5 M NaCl, 20 mM imidazole, 20 mM Tris-HCl pH 8) and sonicated, at 40 Mcycles, 5 times during 30 s at 4 °C in an ice water bath. The extract was centrifuged at 10,000 × g for 1 h at 4 °C to pellet the cellular debris. The supernatant was collected, filtered on a 0.22 µm filter unit (Millex GU, Millipore, Ireland) and applied during 1 h to a 1 mL HisTrap column (GE Healthcare Life Sciences, France), which was pre-equilibrated with 10 mL of binding buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 8). The column was washed with 10 mL of binding buffer. Finally, the His-tagged purified protein was eluted using a 40 mL

linear imidazole gradient varying between 20 and 200 mM. Purified protein was concentrated and equilibrated in 50 mM borate buffer, pH 7.4, using Amicon system (Ultra PL-10, 10,000 NMWL; Millipore, Ireland). Purified protein was concentrated and equilibrated in 50 mM borate pH 7.4 using Amicon system (Ultra PL-10, 10,000 NMWL; Millipore, Ireland). The concentration of the purified protein was calculated using its molar absorbance coefficient (107,260 at 280 nm) with a spectrophotometer (NanoDrop, Thermo Scientific, France).

2.5. SDS-PAGE, protein staining and western blotting

Aliquots of protein extracts or purified proteins (see above) were run under reducing and denaturing conditions on an 8% SDS-PAGE. SDS-PAGEs were stained with silver salts to visualize total or purified proteins [28]. For western blotting, proteins were transferred to a nitrocellulose membrane (0.45 µm, Schleicher & Schuell, Germany) using a semi-dry blotting apparatus (Hoefer scientific instruments, USA). The rat Ap-B was detected with a specific anti-Ap-B polyclonal serum [1] at a dilution of 1:2000. Antigen-antibody complexes were visualized using a goat alkaline phosphatase-conjugated secondary antibody and an NBT-BCIP mixture (Sigma-Aldrich, France).

2.6. Enzyme activity assays

2.6.1. Using chromogenic substrate

Ap-B activity was determined using L-amino acid β -naphthylamide substrates (L-Arg β -NA; Sigma–Aldrich, France). Standard assays were realized at 37 °C for 30 min in 100 mM borate pH 7.4, 150 mM NaCl, containing 0.2 mM L-Arg β -NA. Hydrolysis was interrupted by addition of 60 μ L of freshly prepared solution of Fast Garnet (GBC salt 1 mg/mL in 1 M sodium acetate, pH 4.2, 10% Tween; Sigma–Aldrich, France). The absorbance was read at 535 nm using a microplate spectrophotometer (ASYS UVM340, Austria). For the preliminary screening of the activity of the mutants, tests were realized with 4 μ L of the BugBuster extract (see Production of His-tagged Ap-B in *E. coli* section), which also contains an Ap-N activity. Therefore, the activity assays were also performed with Ap-B (arphamenine A and B, at a 2.5 μ M final concentration) or Ap-N (amastatin, at a 2.5 μ M final concentration) specific inhibitors. The activity with the extract containing wild-type enzyme is used as a reference, and allowed calculating the percentage of activity of the mutants. The use of arphamenine A and B, and of amastatin allows calculating a relative percentage of activity of Ap-B and Ap-N in the wild-type extract, which is compared to that obtained with the different mutant extracts. The average of three independent experimental determinations was calculated, except for Y₂₂₉V/I, due to a weak activity: Y₂₂₉V, Y₂₂₉V- or I-arphamenine B, 13 independent experimental values; Y₂₂₉V-amastatin, 11 values; Y₂₂₉I, Y₂₂₉I-amastatin, 14 values; Y₂₂₉I- or V-arphamenine A, 4 values. Determinations of the kinetic constants of Ap-B were realized in triplicate with 250 ng of purified protein. After 5 min of preincubation of the enzyme at 37 °C in the reaction buffer, the substrate was added at different concentrations (20–300 μ M and the reaction was stopped after 8 min of incubation at 37 °C. The kinetic constants for the enzymatic reaction of Ap-B with L-Arg β -NA were estimated from Lineweaver-Burk plots of 1/v against 1/(S) and Eadie-Hofstee plots of v against v/(S) in the substrate concentration range 0–200 μ M. For the determination of the IC₅₀ of the arphamenine A and B inhibitors with the wild type and the Y₂₂₉H mutant, tests were performed in triplicates with 250 ng of enzyme, different concentrations of inhibitors (between 2 nM and 10 μ M) and 200 μ M of L-Arg β -NA substrate. Assays were realized with a 5 min preincubation at 37 °C without the substrate and an 8 min incubation at 37 °C with the added substrate. Tests of inhibition by arphamenine A and B, bestatin and amastatin on the Y₄₀₉F mutant and the wild type enzyme were realized in the same conditions with 700 ng and 250 ng of protein, respectively. To assess the effects of pH, the activity tests were carried out with 250 ng of enzyme and 200 μ M final of L-Arg β -NA, in a 100 mM phosphate-150 mM NaCl buffer (pH range 6.2–8.3) or in a 100 mM borate-150 mM NaCl buffer (pH range 7.8–8.9). The tests were carried out 8 min at 37 °C.

2.6.2. Using peptide substrate

Enzymatic tests were performed using 600 ng of protein, 5 μ g of Arg₀-Leu₅-enkephalin substrate in a total volume of 100 μ L containing 50 mM Tris–HCl, pH 7.4. The reaction was stopped after 17 h by addition of trifluoroacetic acid (TFA), 0.3% final. The samples were injected on a C18 column (5 μ m diameter; UP5WRP-25K INTERCHROM; Interchim, France) combined with an HPLC (Waters, USA). Peptides are eluted with a gradient of acetonitrile (0.7% TFA) from 20 to 60 % in water (0.1% TFA) with an increment of 1% per minute and a flow rate of 1 ml per minute (total time 40 min).

3. Results

3.1. Identification of conserved tyrosine residues in the M1 family

A multiple sequence alignment of vertebrate proteins belonging to the M1 family of aminopeptidases was performed to establish the basis for site-directed mutagenesis studies. In addition to both signatures, (G/A/H/V) (G/A)MEN and HEXXH¹⁸EX^{80–90}Y, already defined for this family (Fig. 2), several other residues appear to be fully conserved in these sequences. Among them, four tyrosine residues are generally conserved, corresponding to Y₂₂₉, Y₂₈₁, Y₄₁₄ and Y₄₄₁ in the Ap-B sequence (Fig. 2).

This alignment of 273 sequences allows to calculate the percentages of conservation for these residues: Y₂₂₉, 93%; Y₂₈₁, 93%; Y₄₁₄, 100%; Y₄₄₁, 93%. These percentages would be 100% without the sequences of the Ap-O. This protein appears to be an exception in the M1 family, and is probably an example of convergent evolution.

Indeed, Ap-O has about 20–25% sequence identity with the Ap-B and LTA₄H, a percentage that drops to 17–18% with the other family members. Its primary structure retains the HEXXH¹⁸EX^{80–90}Y motif, but lost the (G/A/H/V) (G/A)MEN, wherein only methionine is conserved. Ap-O sequences were thus deleted from the alignment allowing to obtain four fully conserved tyrosine residues in the M1 family. A simplified alignment presenting only the human proteins is shown in Fig. 2. We also focused our attention on a fifth tyrosine residue (Ap-B, Y₄₀₉) that is conserved in the Ap-B and most of the LTA₄H vertebrate sequences, and replaced by a phenylalanine residue in the other aminopeptidase sequences, except for RNPEP-L1 (M vs. Y or F). The interest for Y₄₀₉ is linked to its proximity with Y₄₁₄. Some other tyrosine residues are specifically conserved in the sequences of Ap-B, LTA₄H and RNPEP-L1, such as the Y₂₈₆, Y₃₅₂ and Y₄₂₃ Ap-B residues (Fig. 2). These amino acids should also deserve special attention in the future.

3.2. Site-directed mutagenesis and enzymatic activity of the mutants

The Y₂₂₉, Y₂₈₁, Y₄₁₄ and Y₄₄₁ residues in the Ap-B primary structure were subjected to site-directed mutagenesis. The Y₄₀₉F substitution was also performed. All recombinant proteins were expressed in the Bli5 strain of *E. coli* and mutants retaining enzymatic activity were purified. In all cases, the SDS-PAGEs of purified products showed a major band corresponding to wild type Ap-B, as observed after using western blotting with the Y₂₂₉H mutant, chosen as an example on Fig. 3.

A crude homogenate of *E. coli* was used to estimate the relative enzymatic activity of the wild-type Ap-B and of each different mutant (Fig. 4). Assays were realized with L-Arg β -NA as a substrate and the resulting activity is due to both the presence of Ap-B and of endogenous Ap-N that is also expressed in the crude extracts (Fig. 4, first column of each different protein). A second series of tests was performed in the presence of the Ap-N inhibitor amastatin to estimate the Ap-B activity (Fig. 4, column Ap-B). Conversely, two other series of tests were also realized in the presence of arphamenine A and B, both Ap-B inhibitors, to estimate the level of Ap-N activity (Fig. 4, columns Ap-N-1 and Ap-N-2, respectively). This first step is used to identify mutations, which show a modified activity relative to that of the wild-type enzyme. The resulting activity has to be sufficient and stable to allow the purification and the analyze of the catalytic parameters of the “mutated enzyme.”

As seen in Fig. 4, the aminopeptidase activity of the wild type extract is essentially due to Ap-B [about 90% of Ap-B activity]. Except for Y₂₂₉H (about 80% of Ap-B activity) and partially for Y₄₄₁F (about 25% of Ap-B activity), the other mutations dramatically

Fig. 3. Expression and purification of recombinant wild type Ap-B and Y₂₂₉H proteins. (A) Y₂₂₉F and wild type His-tagged Ap-B were expressed in *E. coli* and purified using Ni-NTA column. One μ g of each sample were run on an 8% SDS-PAGE and stained with silver salts. (B) A similar SDS-PAGE was transferred to a nitrocellulose membrane and Ap-B was detected with a specific anti-Ap-B polyclonal antibody [1]. A narrow band of lower molecular weight is detectable on the silver stained gel and absent on the western blot. This band is typically observed in the experiments of Ap-B purification and it would correspond to a residual presence of GroEL. Relative molecular masses are indicated in kilo Daltons near the prestained molecular mass markers (M).

Table 1

Catalytic parameters of wild type Ap-B and of Y₂₂₉F and Y₂₂₉H mutant towards L-Arg β -NA substrates.^a

	K_m [μ M]	k_{cat} [s^{-1}]	$k_{cat}/K_m \times 10^6$ [$M^{-1} s^{-1}$]
Wild type	162 ± 37	25 ± 4	0.154
Y ₂₂₉ H	60 ± 6.7	9.4 ± 3.6	0.157
Y ₄₀₉ F	98.7 ± 17	4.8 ± 1	0.048
Y ₄₄₁ F	45.3 ± 7.6	2.3 ± 0.2	0.051

^a The results (K_m and k_{cat}) are the mean of three experimental points from different independent experiments (2 for wild type Ap-B, 3 for Y₂₂₉H, 2 for Y₄₀₉F and 3 for Y₄₄₁F). The standard deviation (SD) is calculated.

affect Ap-B activity. Indeed, no significant enzymatic activity was observed with the Y₂₂₉F/V/I/L, Y₂₈₁F and Y₄₁₄ F/H/S/R mutations (Fig. 4; Table 1). The residual activity of the Y₄₀₉F mutant seems to be less sensitive to arphamenine A and B. Contrary to Y₄₁₄H, Y₂₂₉H substitution does not affect the activity and the inhibitor sensitivity of the mutated Ap-B.

These results show that the hydroxyl groups of these tyrosine residues are necessary for the enzyme activity, since the mutation of tyrosine to phenylalanine is deleterious for Y₄₁₄F activity and affects to a lesser extend the activities of Y₂₂₉F, Y₂₈₁F, Y₄₀₉F and Y₄₄₁F. Interestingly, in Y₂₂₉H, but not in Y₄₁₄H, the histidine imidazole group could replace the tyrosine phenol group.

A residual Ap-B activity could sometimes be observed in the crude *E. coli* extracts containing the mutants Y₂₂₉F, Y₂₈₁F, Y₄₀₉F, Y₄₁₄H and Y₄₄₁F. These mutants were purified in order to determine their activity and to characterize their catalytic parameters. However, the activities of these purified mutants are difficult to detect and labile over time, particularly for Y₂₂₉F, Y₂₈₁F and Y₄₁₄H.

Consequently, we focused our efforts on the Y₂₂₉H, Y₄₀₉F and Y₄₄₁F that exhibit a significantly higher and stable activity. Interestingly, the Y₂₂₉H mutation seems to preserve the activity towards

Fig. 4. Relative percentages of enzymatic activity between wild-type Ap-B and mutants. (Wild-type enzyme, Wt, or Mutation name, e.g. Y₂₂₉H), total activity without inhibitor; (Ap-B), remaining activity with amastatin, an Ap-N inhibitor; (Ap-N-1) and (Ap-N-2), remaining activity with arphamenine A and B, respectively, two inhibitors of Ap-B. The columns corresponding to each different protein were shown with alternating gray. Error bars represent the standard deviation between values (see 2.6.1 in Materials and methods section). Y₂₂₉ TAT codon was mutated in TTT (F), CAT (H), TTA (L), GTT (V) and ATA (I). Y₂₈₁ TAT codon was mutated in TTT (F). Y₄₁₄ TAC codon was mutated in TTT (F), CAC (H), TCA (S) and AGA (R). Y₄₄₁ TAT codon was mutated in TTT (F).

Fig. 5. Percentages of activity of the wild type Ap-B (Wt) and Y₂₂₉H with different concentrations of arphamenine A (●) or B (Δ).

L-Arg β-NA. However, compared to the wild type, an increase of the affinity (from 1.9 to 3.7 depending on the SD) and a decrease of the k_{cat} is observed (from 1.6 to 5; Table 1). On the other hand, the catalytic efficiency is altered in the Y₄₀₉F and Y₄₄₁F mutants whose catalytic constant is decreased by a factor about 5 and 10, respectively (Table 1).

The activities of wild type Ap-B and of Y₂₂₉H enzyme were tested in the presence of increasing concentrations of arphamenine A and B inhibitors, which are analogous of Arg-Phe and Arg-Tyr dipeptides, respectively. As observed on Fig. 5, the IC₅₀ are not significantly different between Ap-B and Y₂₂₉H. As these inhibitors are competitive, the formula $K_i = IC_{50}/(1 + S/K_m)$ allows to calculate a K_i , which is, for the arphamenine A, 8.5 nM for wild type Ap-B vs. 7.6 nM for Y₂₂₉H, and for the arphamenine B, 10.3 nM for wild type Ap-B vs. 8.5 nM for Y₂₂₉H.

Similar tests with Y₄₀₉F showed that this mutant has lost its sensitivity towards arphamenine A and B (Fig. 6A) and towards the bestatin inhibitor (Fig. 6B). No significant difference could be observed with amastatin (Fig. 6B), an Ap-N inhibitor that has no effect on Ap-B and Y₄₀₉F enzyme activities.

3.3. Test of activity of the Y₂₂₉H enzyme with an Arg₀-Leu₅-enkephalin peptide substrate

To evaluate the influence of the Y₂₂₉H mutation on the enzyme activity towards a physiological substrate, enzymatic assays were performed using the Arg₀-Leu₅-enkephalin hexapeptide. The HPLC elution profile shows that the Y₂₂₉H enzyme is able to hydrolyze this substrate to generate the Leu₅-enkephalin product (Fig. 7). A MALDI mass spectrometric analysis of the elution products

confirmed the presence of the corresponding substrate (peak 1, Arg₀-Leu₅-enkephalin) and product (peak 2, Leu₅-enkephalin), with a respective molecular mass of 712 Da and 556 Da (data not shown).

3.4. Effect of pH on the activity of Y₂₂₉H compared to that of the wild type enzyme

Tyrosine residues are able to establish H-bonds with their hydroxyl function. Substitution of tyrosine by a histidine residue maintains this ability to form hydrogen bonds via the nitrogen in ϵ or δ position. Since these amino acids have very dissimilar pKa, the effect of pH on the activity of Y₂₂₉H was tested over a pH range from 5.7 to 9.2 and compared to that of the wild type enzyme (Fig. 8). The pH dependence of Y₂₂₉H is close to that of the wild type enzyme, except for a small shift towards higher pH in the descending part of the curve, which provokes a slight modification of the pH optimum activity, i.e. around 7.6 for Y₂₂₉H and around 7.4 for the wild type enzyme. This showed that the Y₂₂₉H mutation does not affect significantly the pH dependence of the enzyme and suggests that the deprotonated form of the histidine could substitute the tyrosine.

4. Discussion

To highlight important amino acids implicated in the structure and/or function of the M1 family aminopeptidases, we focused our attention on four particularly well conserved tyrosine residues, corresponding to Y₂₂₉, Y₂₈₁, Y₄₁₄ and Y₄₄₁ of Ap-B. Our interest also focused on a tyrosine residue existing in Ap-B (Y₄₀₉) and most of

Fig. 6. Effect of different inhibitors on the activity of the wild type and Y₄₀₉F enzymes. (A) Percentage of activity of Ap-B (empty symbol) and Y₄₀₉F (filled symbol) with different concentrations of arphamenine A (○ or ●) or B (Δ or ▲). (B) Percentage of activity of Ap-B (empty symbol) and Y₄₀₉F (filled symbol) with different concentrations of bestatin (□ or ■) or amastatin (○ or ●). The IC₅₀ are between 22 to 29 nM and 4–9 nM for the arphamenine A and B, respectively, and between 165 and 314 nM for bestatin.

Fig. 7. HPLC profile showing the enzymatic activity of Y₂₂₉H towards Arg₀-Leu₅-enkephalin substrate. Absorbance was monitored at 220 nm. The gradient of acetonitrile was indicated as a percentage (blue line). Peak 1 corresponds to the Arg₀-Leu₅-enkephalin substrate and peak 2 to the Leu₅-enkephalin product.

the LTA₄H sequences, but replaced by a phenylalanine residue in most of the other M1 sequences.

In this study, site-directed mutagenesis was performed to substitute tyrosine in order to analyze the importance of the hydroxyl group in the enzymatic mechanism. The importance of the Y₄₁₄ residue is confirmed, since the Y₄₁₄F mutation abolished the enzymatic activity towards L-Arg β-NA. An expected result, since its positional counterpart in the structure of LTA₄H is suspected to take part in the polarization of the carbonyl of the hydrolyzed peptide bond. The analysis of the Y₂₂₉F mutant also showed that the catalysis process is seriously impaired. The effects on the activity using a L-Arg β-NA substrate are less pronounced with the Y₄₀₉F, Y₂₈₁F and Y₄₄₁F mutants. However, their activity remains unstable.

4.1. Tyrosine 229

According to the results obtained with the Y₂₄₄F mutant of the LTA₄H of *S. cerevisiae* [31], it was postulated that this tyrosine stabilizes the transition state of the peptidase activity. The determination of the 3D structure of different aminopeptidases allows

defining more precisely the putative function of this tyrosine that corresponds to Y₂₂₉ in Ap-B. Moreover, based on the 3D structure of an LTA₄H[E296Q] mutant in complex with an RAR substrate [12], a 3D Ap-B model with the RAR peptide docked in the active site was obtained (Fig. 9) [16] that shows that the hydroxyl group of Y₂₂₉ does not interact directly with the substrate, but can contract a hydrogen bond with E₃₀₁. This essential residue, located at the end of a β strand defined by residues of the GAMEN motif, interacts with the NH³⁺-terminus of the substrate (Fig. 9).

Thus, a hydrogen bond implicating the hydroxyl of Y₂₂₉ to stabilize E₃₀₁ seems to be essential for the catalysis. Y₂₂₉ appears to be a crucial actor in the reaction mechanism related to aminopeptidase function. In agreement, the mutation of Y₂₂₉ into a hydrophobic residue, such as Y₂₂₉L/I/V, abolishes the enzyme activity.

Fig. 9. Schematic representation showing the E₃₀₁ residue in the active site of the Ap-B. The Zn²⁺ cation is shown as a purple sphere. E₃₀₁ interacts with the NH₂-terminus of the RAR peptide substrate (3.11 Å distance) and with the hydroxyl group of the Y₂₂₉ residue (1.76 Å distance). The Y₄₁₄ residue is in proximity of the hydrolyzed peptide bond (2.23 Å). The proximity between Y₄₁₄ and Y₄₀₉ is shown (1.84 Å distance). The RAR tripeptide has been docked by superposition of the Ap-B and LTA₄H (pdb: 3b7t) structures with the DaliLite software of the Thornton group at the EMBL-EBI [16]. Images of structures were performed using the Visual Molecular dynamics (VMD) software. Hydrogen bonds (dotted line) and distances are specified.

Fig. 8. Effect of pH on enzyme activity. The percentages of activity of Ap-B (filled symbol) and of Y₂₂₉H (empty symbol) were determined in a phosphate buffer (round) or in a borate buffer (triangle) containing 150 mM NaCl.

As histidine has a steric hindrance similar to that of tyrosine residue and is susceptible to Hp- π interactions [29], the Y229H mutant was generated to test the functional equivalence of the respective side chains at this position. The catalytic efficiency towards L-Arg β -NA is not significantly modified. Thus, an imidazole group could substitute the phenol group in a defined amino acid environment. Inhibition by arphamenine A and B, reported to be specific inhibitors of Ap-B, is the same as that of the wild type enzyme. Moreover, the Y229H mutant is able to hydrolyze the peptide bond on the carboxylic side of the arginine residue of Arg0-Leu5-enkephalin. The properties of this mutant are thus similar to those of the wild type, including the sensitivity to arphamenine A and B and the 2.5-fold increase of activity towards L-Arg β -NA in the presence of 150 mM NaCl (data not shown). In addition, circular dichroism analysis of this mutant compared to the wild type enzyme shows no significant difference of secondary structure (data not shown). The pH dependence of Y229H is close to that of the wild type enzyme, suggesting that the deprotonated form of the histidine could substitute the tyrosine. Moreover, Y229 and E301 could also establish hydrogen bonds with a conserved asparagine residue (2.17 and 3.49 Å distance, respectively; Fig. 10). For ERAP2, an atomic interaction (3.1 Å) has notably been observed between the corresponding residues Y262 and N392 [21]. N392 (Ap-B, N347) was mapped in the active site, in proximity of significant residues for catalysis such as E200 (Ap-B, Q171), E337 (Ap-B, E301) or E393 (Ap-B, E348). Since, the natural N392K polymorphism of ERAP2 was linked with a predisposition to several human diseases, such as ankylosing spondylitis or preeclampsia [32,33]. A recent study showed that this N392K substitution interferes with the stabilization of the NH₂-terminus of the substrate, with an effect on the catalytic turnover (k_{cat}), the substrate specificity, but not the affinity of ERAP2 towards its substrate [34]. Therefore, these observations are

Fig. 10. Hydrogen binding network between Y229, E301, N347 residues and the NH₂-terminus of P1 of the RAR tripeptide substrate. Hydrogen bonds and distances are specified between N347 and Y229 (2.17 Å), N347 and E301 (3.49 Å), Y229 and E301 (3.11 Å). Images of structures were performed using the Visual Molecular dynamics (VMD) software.

in agreement with our results, which show that the integrity and the environment of Y229 and E301 residues of Ap-B are necessary for aminopeptidase activity.

4.2. Tyrosine 414

Y414F/A/H/L/R/S mutations led to inactive enzymes. Therefore, the Y414H replacement could not substitute the particular properties of the Y414 residue. Contrary to Y229H, the histidine residue of Y414H is not in the same environment. One possible explanation could be the proximity between Y414 and the Zinc atom (4.11 Å, not shown). The Y414H mutation may indeed disturb the enzymatic reaction because this histidine residue could potentially act as another chelator of the Zn²⁺ catalytic atom. Similar results are observed for LTA₄H, the corresponding Y383H/F/Q mutants being devoid of peptidase activity [35].

4.3. Tyrosine 409

The hydroxyl group of Y409 could potentially establish hydrogen bonds with Y414 (Fig. 11). Y409F mutation affects the enzyme activity toward L-Arg β -NA, as the enzyme turnover is decreased by a factor of 5.5–6. Moreover, Y409F has lost its sensitivity towards arphamenine A and B, as well as bestatin. The analysis of the 3D Ap-B model indicates that Y409 is essential for the role of Y414 (Fig. 11). Indeed, the proximity (1.84 Å) suggests that Y409 strengthens the induced polarization of the peptide bond by Y414. However, the corresponding Y378F mutation in LTA₄H reduces the peptidase activity by 57%, using L-Ala 4-nitroanilide. Consequently, the affinity, but not the enzyme turnover, is affected [36]. Moreover, the epoxide hydrolase activity of Y378F produced, in addition to Δ^6 -*cis*- Δ^8 -*trans* LTB₄ (70–80%), a second metabolite, the Δ^6 -*trans*- Δ^8 -*cis* LTB₄ (20–30%) [36]. In some vertebrate, LTA₄H has a phenylalanine residue instead of a tyrosine at the corresponding position. This is the case for the *X. laevis* LTA₄H that produces this second metabolite [37]. The production of this LTB₄ isomeric compound is abolished by the corresponding F375Y mutation (human LTA₄H, Y378; Ap-B, Y409) [38]. A role for the Y375 hydroxyl group in the stabilization by a hydrogen bond of the Y380 catalytic residue (Ap-B, Y414) was

Fig. 11. Hydrogen bond interactions between the Y414 catalytic tyrosine and the Y409 of Ap-B superposed with that of corresponding amino acids of the human Ap-N (Y477 and F472; 4FYQ). A distance of 1.84 Å is determined for the hydrogen bond between Y409 and Y414. Images of structures were performed using the Visual Molecular dynamics (VMD) software.

thus proposed [38]. From all of these observations, a question arises and for which a response will be probably brought in the future: Is the hydroxyl group of the tyrosine residue (Ap-B, Y₄₀₉) necessary for the hydrolysis of peptides by the M1 aminopeptidases with three structural domains?

Y₄₀₉ is replaced by a phenylalanine in some LTA₄H sequences and in all primary structures of aminopeptidases with four domains (e.g. Ap-N; Fig. 1B). The supplementary domain governs the existence of closed and open conformations. The superposition of the Ap-B 3D model with the closed conformation of the Ap-N crystallographic structure (pdb: 4FYQ) shows an inverse orientation of the F₄₇₂ residue of Ap-N compared to the corresponding Y₄₀₉ of Ap-B (Fig. 11). F₄₇₂ is farthest from the catalytic Y₄₇₇, which could promote the mobility of this catalytic tyrosine as observed for closed and open structures of the ERAP1 and ERAP2 aminopeptidases [24]. Consequently, other experiments, especially with different peptide substrates, will be necessary to highlight the subtle differences governing the role of the tyrosine vs. phenylalanine in the M1 aminopeptidase catalytic mechanisms.

4.4. Tyrosine 281

Both Y₂₈₁ and Y₄₄₁ are located rather near the surface of Ap-B. In contrast to Y₂₂₉ and Y₄₁₄, which have a concerted function in the catalytic mechanism of enzymes of the M1 family, it is difficult to evaluate whether Y₂₈₁ and Y₄₄₁ establish direct or indirect interactions with the substrate or with amino acids present in the first shell of the active site.

The Y₂₈₁F mutation affects significantly the enzyme activity, that drops rapidly and catalytic parameters could not be obtained. The role of this residue could be a contribution to the maintenance of a spatial arrangement important for the function of some catalytic amino acids. Notably, as Y₂₈₁ is in proximity of E₃₀₁ residue in the GAMEN motif, it can be of interest to analyze the amino acid environment and its structural influence. As observed on the model of the Ap-B (Fig. 12), the hydroxyl group of Y₂₈₁ is able to establish a hydrogen bond with the carbonyl of R₂₈₅. This latter residue is conserved in the sequences of Ap-B and RNPEP-L1 but it is replaced

by a glutamine in LTA₄H and by a lysine in the other aminopeptidases. Y₂₈₁ and R₂₈₅ are located on a loop in proximity of a β sheet, which expose E₃₀₁ in the catalytic site (Fig. 12). This H-bond could participate to the stabilization of this functional β sheet. Other chemical interactions form a network that could stabilize this functional structure. Especially, an ionic interaction between R₂₈₅ and D₂₈₇ (a residue conserved in all sequences) is identifiable, as well as an aromatic interaction between Y₂₈₁ and W₂₈₃ in the aminopeptidases with three domains. In the aminopeptidases with four domains, this interaction is observed with the tryptophan residue located just after the (G/A/H/V) (G/A)MEN motif (Fig. 2).

In addition, R₂₈₅ in Ap-B could also be implicated in a cation π interaction with Y₂₈₁, upon a reorientation of its side chain resulting from the tilt of the loop to which it belongs. This dynamic process could contribute to the conformational arrangement of the catalytic site. Other mutagenesis experiments and structural analyses will be needed to verify the role of Y₂₈₁ in important structural and functional interactions necessary for the enzyme catalytic mechanism.

4.5. Tyrosine 441

The Y₄₄₁F mutation reduces by 66% the catalytic capacity of the Ap-B. The environment of Y₄₄₁ is highly hydrophobic with possibility of stacking with numerous phenylalanine residues (not shown). Moreover, a hydrogen bond can be suspected between the hydroxyl group of Y₄₄₁ and the carbonyl of D₄₅₄ (3.63 Å; Fig. 13). This aspartate residue seems to be important, since it is conserved in the sequences of the vertebrate aminopeptidases of the M1 family (LTA₄H, D₄₂₃).

For these aminopeptidases, it appears that the strength of the hydrogen bond network established with this tyrosine residue could vary depending on the environment. Notably for LTA₄H, the hydrogen bond cannot be visualized between the hydroxyl group of Y₄₁₀ and the carbonyl of D₄₂₃ but with the nitrogen of H₃₂₀ (4.16 Å; Fig. 13), a histidine residue that is generally replaced by a phenylalanine in other aminopeptidases (Fig. 2). Such an interaction could indirectly participate to the arrangement of the Zn²⁺ catalytic atom in the active site. Indeed, H₃₂₀ is located in proximity of E₃₁₈, one of the essential residues of the active site as chelator of the Zn²⁺ atom.

Thus in the M1 family of aminopeptidases, the role of Y₄₄₁ could be in relation with a difference in substrate specificity and/or in catalytic efficiencies. Other more detailed studies, in particular

Fig. 12. Proximity and chemical interactions between Y₂₈₁ and R₂₈₅. A salt bridge could be established between R₂₈₅ and D₂₈₇. These interactions could participate to the stabilization of the small beta sheet that exposes the E₃₀₁ residue in the active site of the Ap-B. A distance of 1.75 Å is determined for the hydrogen bond between the hydroxyl of Y₂₈₁ and the carbonyl of R₂₈₅. Images of structures were performed using the Visual Molecular dynamics (VMD) software.

Fig. 13. Superposition of the structures of Ap-B and LTA₄H (pdb: 3B7T) showing a potential hydrogen bond between the hydroxyl group of Y₄₄₁ and the carbonyl of D₄₅₄ (3.63 Å, Ap-B). In LTA₄H, no such hydrogen bond between Y₄₁₀ and D₄₂₃ is identified, on the other hand this bond could be established with H₃₂₀ (4.16 Å; Ap-B, F₃₅₀). Images of structures were performed using the Visual Molecular dynamics (VMD) software.

molecular dynamics simulations, will be necessary to understand the complex role of this tyrosine residue in the enzymatic reaction of each aminopeptidases of the M1 family.

In conclusion, this work highlights the essential function of residues from second shell and of amino acids distant from the active site in enzyme catalysis. In particular, the tyrosine residues of which the hydroxyl groups forming hydrogen bonds are necessary for the enzymatic catalysis. Since these enzymes are involved in important physiological roles, this approach should help to the conception of highly specific inhibitors that could be used in a therapeutic research pathway.

Conflict of interest

There is no conflict of interest.

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References

- [1] S. Cadel, A.R. Pierotti, T. Foulon, C. Créminon, N. Barré, D. Segrétain, P. Cohen, Aminopeptidase B in the rat testes: isolation, functional properties and cellular localization in the seminiferous tubules, *Mol. Cell. Endocrinol.* 110 (1995) 149–160.
- [2] T. Foulon, S. Cadel, V. Chesneau, M. Draoui, A. Prat, P. Cohen, Two novel metallopeptidases with a specificity for basic residues. Functional properties, structure and cellular distribution, *Ann. N. Y. Acad. Sci.* 780 (1996) 96–106.
- [3] C. Piesse, M. Tymms, E. Garrafa, C. Gouzy, M. Lacasa, S. Cadel, P. Cohen, T. Foulon, Human aminopeptidase B [rnp] on chromosome 1q32.2: complementary DNA, genomic structure and expression, *Gene* 292 (2002) 129–140.
- [4] C. Piesse, S. Cadel, C. Gouzy-Darmon, J.C. Jeanny, V. Carrière, D. Goidin, L. Jonet, D. Gourdj, P. Cohen, T. Foulon, Expression and cellular distribution of aminopeptidase B in the developing and adult rat retina, *Exp. Eye Res.* 79 (2004) 639–648.
- [5] G. Fontes, A.D. Lajoix, F. Bergeron, S. Cadel, A. Prat, T. Foulon, R. Gross, S. Dalle, D. Le-Nguyen, F. Tribillat, D. Bataille, Miniglucagon-generating endopeptidase, which processes glucagon in miniglucagon is composed of NRD convertase and aminopeptidase B, *Endocrinology* 146 (2005) 702–712.
- [6] M.C. Beinfeld, L. Funkelstein, T. Foulon, S. Cadel, K. Kitagawa, T. Toneff, T. Reinheckel, C. Petres, V. Hook, Cathepsin L plays a major role in cholecystokinin production in mouse brain cortex and in pituitary AtT20-cells: protease gene knockout and inhibitor studies, *Peptide* 30 (1) (2009) 882–1891.
- [7] S.R. Hwang, A. O'Neill, S. Bark, T. Foulon, V. Hook, Secretory vesicle aminopeptidase B related to neuropeptide processing: molecular identification and subcellular localization to enkephalin- and NPY-containing chromaffin granules, *J. Neurochem.* 100 (2007) 1340–1350.
- [8] M. Kouach, B. Desbuquois, F. Authier, Endosomal proteolysis of internalized [ArgA0]-human insulin at neutral pH generates the mature insulin peptide in rat liver in vivo, *Diabetologia* 12 (2009) 2621–2632.
- [9] S. Urban, K. Textoris-Taube, B. Reimann, K. Janek, T. Dannenberg, F. Ebstein, C. Seifert, F. Zhao, J.H. Kessler, A. Halenius, P. Henklein, J. Paschke, S. Cadel, H. Bernhard, F. Ossendorp, T. Foulon, D. Schadendorf, A. Paschen, U. Seifert, The efficiency of human cytomegalovirus pp65A95–503 CD8+ T cell epitope generation is determined by the balanced activities of cytosolic and endoplasmic reticulum-resident peptidases, *J. Immunol.* 189 (2012) 529–538.
- [10] S. Cadel, T. Foulon, A. Viron, S. Midol-Monnet, A. Balogh, N. Noel, P. Cohen, Aminopeptidase B from the rat testis is structurally and enzymatically related to leukotriene-A₄ hydrolase, *Proc. Natl. Acad. Sci.* 94 (1997) 2963–2968.
- [11] N.M. Hooper, Families of zinc metalloproteases, *FEBS Lett.* 354 (1994) 1–6.
- [12] F. Tholander, A. Muroya, B.P. Roques, M.C. Fournié-Zaluski, M.M. Thunnissen, J.Z. Haeggström, Structure-based dissection of the active site chemistry of leukotriene A₄ hydrolase: implications for M1 aminopeptidases and inhibitor design, *Chem. Biol.* 15 (2008) 920–929.
- [13] G. Vazeux, X. Iturriz, P. Corvol, C. Llorens-Cortes, A glutamate residue contributes to the exopeptidase specificity in aminopeptidase A, *Biochem. J.* 334 (1998) 407–413.
- [14] X. Iturriz, R. Rozenfeld, A. Michaud, P. Corvol, C. Llorens-Cortes, Study of asparagine 353 in aminopeptidase A: characterization of a novel motif [GXMEN] implicated in exopeptidase specificity of monozinc aminopeptidases, *Biochemistry* 40 (2001) 14440–14448.
- [15] P.C. Rudberg, F. Tholander, M. Thunnissen, J.Z. Haeggström, Leukotriene/aminopeptidase. Glutamate 271 is a catalytic residue with specific roles in two distinct enzyme mechanisms, *J. Biol. Chem.* 277 (2002) 1398–1404.
- [16] V.L. Pham, C. Gouzy-Darmon, J. Pernier, C. Hanquez, V. Hook, M.C. Beinfeld, P. Nicolas, C. Etchebest, T. Foulon, S. Cadel, Mutation in the substrate-binding site of aminopeptidase B confers new enzymatic properties, *Biochimie* 4 (2011) 730–741.
- [17] L. Chen, Y.L. Lin, G. Peng, F. Li, Structural basis for multifunctional roles of mammalian aminopeptidase N, *Proc. Natl. Acad. Sci. U. S. A.* 109 (2012) 17966–17971.
- [18] M.M. Thunnissen, P. Nordlund, J.Z. Haeggström, Crystal structure of human leukotriene A₄ hydrolase, a bifunctional enzyme in inflammation, *Nat. Struct. Biol.* 8 (2001) 131–135.
- [19] A. Addlagatta, L. Gay, B.W. Matthews, Structure of aminopeptidase N from *Escherichia coli* suggests a compartmentalized, gated active site, *Proc. Natl. Acad. Sci.* 103 (2006) 13339–13344.
- [20] B. Nocek, R. Mulligan, M. Bargassa, F. Collart, A. Joachimiak, Crystal structure of aminopeptidase N from human pathogen *Neisseria meningitidis*, *Proteins* 70 (2008) 273–279.
- [21] S. McGowan, C.J. Porter, J. Lowther, C.M. Stack, S.J. Golding, T.S. Skinner-Adams, K.R. Trenholme, F. Teuscher, S.M. Donnelly, J. Grembecka, A. Mucha, P. Kafarski, R. Degori, A.M. Buckle, D.L. Gardiner, J.C. Whisstock, J.P. Dalton, Structural basis for the inhibition of the essential Plasmodium falciparum M1 neutral aminopeptidase, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 2537–2542.
- [22] A.H. Wong, D. Zhou, J.M. Rini, The X-ray crystal structure of human aminopeptidase N reveals a novel dimer and the basis for peptide processing, *J. Biol. Chem.* 287 (2012) 36804–36813.
- [23] T.T. Nguyen, S.C. Chang, I. Evnouchidou, I.A. York, C. Zikos, K.L. Rock, A.L. Goldberg, E. Stratikos, L.J. Stern, Structural basis for antigenic peptide precursor processing by the endoplasmic reticulum aminopeptidase ERAP1, *Nat. Struct. Mol. Biol.* 18 (2011) 604–613.
- [24] J.R. Birtley, E. Saridakis, E. Stratikos, I.M. Mavridis, The crystal structure of human endoplasmic reticulum aminopeptidase 2 reveals the atomic basis for distinct roles in antigen processing, *Biochemistry* 51 (2012) 286–295.
- [25] A. Wetterholm, J.Z. Haeggström, Leukotriene A₄ hydrolase: an anion activated peptidase, *Biochim. Biophys. Acta* 1123 (1992) 275–281.
- [26] R.J. Snelgrove, P.L. Jackson, M.T. Hardison, B.D. Noerager, A. Kinloch, A. Gaggari, S. Shastry, S.M. Rowe, Y.M. Shim, T. Hussell, J.E. Blalock, A critical role for LTA₄H in limiting chronic pulmonary neutrophilic inflammation, *Science* 330 (2010) 90–94.
- [27] R.J. Snelgrove, Leukotriene A₄ hydrolase: an anti-inflammatory role for a proinflammatory enzyme, *Thorax* 66 (2011) 550–551.
- [28] V.L. Pham, M.S. Cadel, C. Gouzy-Darmon, C. Hanquez, M.C. Beinfeld, P. Nicolas, C. Etchebest, T. Foulon, Aminopeptidase B, a glucagon-processing enzyme: site directed mutagenesis of the Zn²⁺ binding motif and molecular modeling, *BMC Biochem.* 8 (2007) 21.
- [29] Q.S. Du, Q.Y. Wang, L.Q. Du, D. Chen, R.B. Huang, Theoretical study on the polar hydrogen-pi [Hp-pi] interactions between protein side chains, *Chem. Cent. J.* 7 (2013) 92.
- [30] M. Andberg, A. Wetterholm, J.Z. Haeggström, Mutation of Tyr-383 in leukotriene A₄ hydrolase: effects on enzyme activities, *Adv. Exp. Med. Biol.* 433 (1997) 149–152.
- [31] M.W. Thompson, E.D. Archer, C.E. Romer, R.L. Seipelt, A conserved tyrosine residue of *Saccharomyces cerevisiae* leukotriene A₄ hydrolase stabilizes the transition state of the peptidase activity, *Peptides* 27 (2006) 1701–1709.
- [32] F.W. Tsui, N. Haroon, J.D. Reveille, P. Rahman, B. Chiu, H.W. Tsui, R.D. Inman, Association of an ERAP1 ERAP2 haplotype with familial ankylosing spondylitis, *Ann. Rheum. Dis.* 69 (2010) 733–736.
- [33] M.P. Johnson, L.T. Roten, T.D. Dyer, C.E. East, S. Forsmo, J. Blangero, S.P. Brennecke, R. Austgulen, E.K. Moses, The ERAP2 gene is associated with pre-eclampsia in Australian and Norwegian populations, *Hum. Genet.* 126 (2009) 655–666.
- [34] I. Evnouchidou, J. Birtley, S. Seregin, A. Papakyriakou, E. Zervoudi, M. Samiotaki, G. Panayotou, P. Giastas, O. Petrakis, D. Georgiadis, A. Amalfitano, E. Saridakis, I.M. Mavridis, E. Stratikos, A common single nucleotide polymorphism in endoplasmic reticulum aminopeptidase 2 induces a specificity switch that leads to altered antigen processing, *J. Immunol.* 189 (2012) 2383–2392.
- [35] M. Blomster, A. Wetterholm, M.J. Mueller, J.Z. Haeggström, Evidence for a catalytic role of tyrosine 383 in the peptidase reaction of leukotriene A₄ hydrolase, *Eur. J. Biochem.* 231 (1995) 528–534.
- [36] M.J. Mueller, M.B. Andberg, B. Samuelsson, J.Z. Haeggström, Leukotriene A₄ hydrolase, mutation of tyrosine 378 allows conversion of leukotriene A₄ into an isomer of leukotriene B₄, *J. Biol. Chem.* 271 (1996) 24345–24348.
- [37] F. Strömberg, M. Hamberg, U. Rosenzvist, S.E. Dahlén, J.Z. Haeggström, Formation of a novel enzymatic metabolite of leukotriene A₄ in tissues of *Xenopus laevis*, *Eur. J. Biochem.* 238 (1996) 599–605.
- [38] A. Stsiapanava, F. Tholander, R.B. Kumar, A.A. Qureshi, D. Niegowski, M. Hasan, M. Thunnissen, J.Z. Haeggström, A. Rinaldo-Matthis, Product formation controlled by substrate dynamics in leukotriene A₄ hydrolase, *Biochim. Biophys. Acta* 1844 (2014) 439–446.