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Research paper

S1 pocket fingerprints of human and bacterial methionine aminopeptidases determined using fluorogenic libraries of substrates and phosphorus based inhibitors

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ABSTRACT

Methionyl aminopeptidases (MetAPs) are metallo-dependent proteases responsible for removing of N-terminal methionine residue of peptides and proteins during protein maturation and activation. In this report we use a comprehensive strategy to screen the substrate specificity of three methionyl aminopeptidases: *Homo sapiens* MetAP-1, *Homo sapiens* MetAP-2 and *Escherichia coli* MetAP-1. By utilizing a 65-membered fluorogenic substrate library consisting of natural and unnatural amino acids we established detailed substrate preferences of each enzyme in the S1 pocket. Our results show that this pocket is highly conserved for all investigated MetAPs, very stringent for methionine, and that several unnatural amino acids with methionine-like characteristics were also well hydrolyzed by MetAPs. The substrate-derived results were verified using several phosphonate and phosphinate-based inhibitors.

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1. Introduction

In all organisms, protein translation mainly starts with a methionine in Eukaryotes and formylated methionine in Prokaryotes. Subsequently, post-translational modifications are required to provide stable and properly localized protein. One of the maturation steps of many proteins is the removal of N-terminal methionine by methionyl aminopeptidases (MetAPs) [1–3]. This family of enzymes is divided into two major classes: type I MetAP (MetAP-1) and type II MetAP (MetAP-2), the latter containing an additional domain of 60 amino acids [4]. MetAP-1 and MetAP-2 are found in Eukaryotes, while in Prokaryotes such as *Escherichia coli*, *Salmonella typhimurium* or *Bacillus subtilis* only MetAP-1 is found [5]. In contrast, archaea contain only the MetAP-2 class. MetAP-1 in various organisms can further be divided into 3 subclasses: type Ia, Ib and Ic. For example, *E. coli* possess a type *Ia*MetAP with only a catalytic domain [6]. In *Homo sapiens*, on the other hand, there is MetAP-1 (type *Ib*), which contains three domains: a zinc fingers domain, 50-residue linker and catalytic domain. Type *Ib* is longer than type *Ia* by about extra 120 amino acids. Contrary to MetAP-1, the structure of human MetAP-2 does not have the connector motif, but instead it has a helical insertion in the middle of the catalytic domain, which can play the same role [4,7,8]. These two MetAPs have very similar structures despite low sequence identity. The regions that create the methionine pocket are not well conserved.

Sequence alignments of EcMetAP-1, hMetAP-1 and hMetAP-2 exemplify the divergent resemblance of these proteins. While

Abbreviations: EcMetAP, *Escherichia coli* MetAP-1; hMetAP-1, *Homo sapiens* MetAP-1; hMetAP-2, *Homo sapiens* MetAP-2; ACC, 7-amino-4-carbamoylmethylcoumarin; Nva, norvaline; hLeu, homoleucine; hArg, homoarginine; Abu, 2-aminobutyric acid; hCha, 4-cyclohexyl-L-butyric acid; 3—CN—Phe, 3-cyano-L-phenylalanine; Dap, L-2,3-diaminopropionic acid; Dab, L-2,4-diaminobutyric acid; 1-Nal, 3-(1-naphthyl)-L-alanine; Tic, (3L)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; 2-Nal, 3-(2-naphthyl)-L-alanine; 4-NO_2-Phe, 4-nitro-L-phenylalanine; 4-Cl-Phe, 4-chloro-L-phenylalanine; 6-Ahx, 6-aminohexanoic acid; Phg, L-phenylglycine; Bip, L-biphenylalanine; Bpa, 4-benzoyl-L-phenylalanine; 4-I-Phe, 4-iodo-L-phenylalanine; Cba, L-2-amino-4-cyanobutyric acid; Igl, L-2-indanylglycine; 3-NO_2-Tyr, 3-nitro-L-tyrosine; 4-NH_2-Phe, 4-amino-L-phenylalanine; 4-Br-Phe, 4-bromo-L-phenylalanine; β-Z-Dab, L-2,4-(carbobenzyloxy)-diaminobutyric acid; Nle, norleucine.

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Table 1
Amino acids participating in Co^{2+} coordination and building S1 and S1′ pockets of methionine aminopeptidases.

Co^{2+} coordinating			S1 pocket			S1′ pocket		
EcMetAP-1	hMetAP-1	hMetAP-2	EcMetAP-1	hMetAP-1	hMetAP-2	EcMetAP-1	hMetAP-1	hMetAP-2
Asp97	Asp220	Asp251	Cys59	Pro183	Pro220	Tyr-168	Tyr291	Leu328
Asp108	Asp231	Asp262	Tyr62	Tyr186	Met384	Gln233	Gln457	Gln457
His171	His294	His331	Tyr65	Phe189	His382	Met-206	Met329	Phe366
Glu204	Glu327	Glu364	Cys70	Cys193	Gly222	Glu204	Glu327	Glu364
Glu235	Glu358	Glu459	Phe177	Phe300	Ile338			Leu447
			Trp221	Trp344	Tyr444			

MetAPs of type I from *E. coli* and from humans reveal significant similarity (45% of overall structure), comparison of human type I and II shows extensive differences (17% between EcMetAP-1 and hMetAP-2 and 15% between hMetAP-1 and hMetAP-2) [8,9]. In spite of this, the structure of active site remains similar. It was reported that the five amino acids residues which bind the cobalt ions are conserved in EcMetAP-1 and hMetAP-2 [10], and hMetAP-1 has equivalent residues fulfilling this function.

Residues recognizing substrates and building the S1 and S1′ subsites differ significantly between type I and type II MetAPs (see Table 1) [11]. For example, the S1 pocket of EcMetAP-1 is formed by surface loops (59–68 and 214–224 amino acids residues), which in hMetAP-2 are much shorter and the function is subsumed by another, unique for this enzyme, loop with conserved Tyr444 corresponding to Trp221 of EcMetAP [10,12]. Additional differences exist also in the S1′ pocket, including Leu447 in the loop of hMetAP-2 and Leu328, Phe366 instead of Tyr-168 and Met-206 found in EcMetAP-1. hMetAP-1 has a reduced size of the active site as consequence of location of residues 298–303 about 1Å closer to the active site than it is observed in hMetAP-2 [4]. Despite these differences, the shape of the substrate binding pockets remains similar. This is reflected in substrate preferences of all enzymes for an N-terminal methionine. However, the observed differences also generate an opportunity for the design of specific substrates and inhibitors.

Biological functions of MetAPs are linked to angiogenesis or cell cycle progression. Suppression of formation of new blood vessels is inhibited by the fungal metabolite fumagillin (an efficient inhibitor of MetAP-2) suggesting its crucial role in cancer development [13,14]. Additionally, MetAP-1 was proposed to be a target for anticancer agents due to its function in cell cycle progression. Selective inhibitors of this enzyme restrains cells in the G2 phase of mitosis, which in case of leukemia cells induces apoptosis [15]. In *E. coli* and *S. typhymurium* the role of MetAPs is of vital relevance and therefore inhibitors could become relevant for anti-infective drug development [16].

The precise analysis of substrate binding behavior of EcMetAP-1, hMetAP-1 and hMetAP-2 is a crucial step for further investigation of these enzymes role and can facilitate determination of potential inhibitors. We present a comparison of substrate specificity based on an extensive 65-membered library of highly diverse fluorogenic substrates that covers a broad spectrum of possible interactions in the S1 pocket (according to Schechter and Berger nomenclature [17]) and demonstrate subtle differences between these closely related enzymes. We also present kinetic analysis of phosphonate and phosphinate-based inhibitors of MetAPs based on the optimal substrate selection.

2. Experimental

2.1. Synthesis of a substrate library

A 65-membered library of natural amino acids and derivatives coupled to ACC fluorophore was synthesized using a procedure described earlier [18].

2.2. Synthesis of inhibitors (α-aminoalkylphosphonic acids and α-aminoalkylphosphinic acids)

Compounds **1**–**4** were prepared as described previously [19–22]. The detailed description of synthesis and characterization of compounds **5**–**7** and **10**–**12** is described in the Supplementary Material.

2.3. Enzyme expression

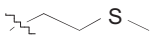
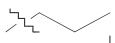
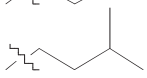
Cloning, expression and purification of the EcMetAP in E. coli

A C-terminal poly-His-tagged *Ec*MetAP was obtained by expression in *E. coli* BL21(DE3) cells (Novagen) using an Arg-175-Gln mutant kindly provided by Prof. W.T. Lowther and Prof. B. Matthews. A detailed description of the preparation based on nickel affinity chromatography is given in reference [23].

Cloning, expression and purification of the hMetAP-1 in Escherichia coli

The gene for *h*MetAP-1 was synthesized as an optimized nucleotide sequence for *E. coli* expression (Geneart) and cloned into the pET-28a(+) vector (Novagen) using *Bam*H1 and *Sac*1 cloning sites. The enzyme with an N-terminal His-tag was overexpressed in

Table 2
Comparision of the kinetic parameters (K_m, k_{cat}, k_{cat}/K_m) of the selected substrates for *Hs*MetAP-1 and *Hs*MetAP-**2**. The results are presented as mean values of three replicates with standard deviation.

Name	Structure	*Homo sapiens* MetAP-1			*Homo sapiens* MetAP-2		
		K_m, μM	$k_{cat}*10^6$, s^{-1}	k_{cat}/K_m, M^{-1} s^{-1}	K_m, μM	$k_{cat}*10^6$, s^{-1}	k_{cat}/K_m, M^{-1} s^{-1}
Met		137.6 ± 4.9	3874 ± 257	28.2 ± 3.0	88.1 ± 12.7	388 ± 42	4.41 ± 0.4
Nva		196.4 ± 9.7	124 ± 13	0.6 ± 0.1	not hydrolyzed		
hLeu		148.3 ± 7.6	2307 ± 203	15.6 ± 1.9	105.1 ± 3.2	118 ± 10	1.13 ± 0.2
Nle		151.3 ± 3.1	1785 ± 77	11.8 ± 0.8	102.9 ± 7.2	71 ± 8	0.7 ± 0.1

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