FEBS Open Bio 5 (2015) 429–436

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

Substrate specificity of mitochondrial intermediate peptidase analysed by a support-bound peptide library

M.F.M. Marcondes, F.M. Alves, D.M. Assis [1], I.Y. Hirata, L. Juliano, V. Oliveira *, M.A. Juliano *

Department of Biophysics, Escola Paulista de Medicina, Universidade Federal de São Paulo, Rua Pedro de Toledo, 669 – 7° andar, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 24 February 2015
Revised 20 April 2015
Accepted 7 May 2015

Keywords:
oct1
Octapeptidyl amino peptidase 1
Peptidase
Mitochondria
FRET libraries
Substrate specificity

ABSTRACT

The substrate specificity of recombinant human mitochondrial intermediate peptidase (*h*MIP) using a synthetic support-bound FRET peptide library is presented. The collected fluorescent beads, which contained the hydrolysed peptides generated by *h*MIP, were sequenced by Edman degradation. The results showed that this peptidase presents a remarkable preference for polar uncharged residues at P_1 and P_1' substrate positions: Ser = Gln > Thr at P_1 and Ser > Thr at P_1'. Non-polar residues were frequent at the substrate P_3, P_2, P_2' and P_3' positions. Analysis of the predicted MIP processing sites in imported mitochondrial matrix proteins shows these cleavages indeed occur between polar uncharged residues. Previous analysis of these processing sites indicated the importance of positions far from the MIP cleavage site, namely the presence of a hydrophobic residue (Phe or Leu) at P_8 and a polar uncharged residue (Ser or Thr) at P_5. To evaluate this, additional kinetic analyses were carried out, using fluorogenic substrates synthesized based on the processing sites attributed to MIP. The results described here underscore the importance of the P_1 and P_1' substrate positions for the hydrolytic activity of *h*MIP. The information presented in this work will help in the design of new substrate-based inhibitors for this peptidase.

1. Introduction

Most mitochondrial proteins are encoded by nuclear DNA and produced by ribosomes outside this organelle [1]. Numerous proteins directed to the mitochondrial matrix, intermembrane space or internal membrane are synthesized with an extended N-terminus, a signal sequence, which is cleaved-off by specific mitochondrial peptidases [2]. The mitochondrial processing peptidase (MPP; EC 3.4.24.64) is the most important processing enzyme that acts on proteins directed to the inner membrane, inter-membrane space or mitochondrial matrix. Most of the proteins targeted to the mitochondrial matrix or inner membrane present a single cleavage by MPP. However, in the mitochondrial matrix, some proteins also take a second sequential cleavage by the mitochondrial intermediate peptidase (MIP; EC 3.4.24.59). In these proteins, MIP removes eight residues from the newly generated N-terminus after the MPP action [3–6].

Human MIP (*h*MIP) is encoded by a nuclear gene (MIPEP), and is transported to the mitochondria. It also presents a signal sequence of 35 residues that is cleaved by MPP. MIP is a soluble monomer of about 75 kDa with the typical zinc ion binding motif, HEXXH [7]. The higher levels of *h*MIP expression were detected in tissues that consume oxygen at a high rate, i.e. in heart and skeletal muscle, and in several regions of the brain [8]. This peptidase was identified in many mammals and other species too [5,9–12]. The enzyme from *Saccharomyces cerevisiae*, formerly known as yeast mitochondrial intermediate peptidase (*y*MIP) but now called octapeptidyl amino peptidase 1 (oct1), is the best characterized mitochondrial intermediate peptidase. According to the N-terminal end rule, the action of oct1 is important to the stability of the oct1 processed proteins in the mitochondrial matrix [13,14]. This hypothesis arises from the observations of the amino terminal sequences from the processed proteins by this enzyme, before and after the oct1 action, and, from the direct analysis of the stability of three known

Abbreviations: Abz, *ortho*-aminobenzoic acid; EDDnp, N-(2,4-dinitrophenyl)-ethylenediamine; FRET, fluorescence resonance energy transfer; *h*MIP, human mitochondrial intermediate peptidase; HOBt, hydroxybenzotriazole; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; NMM, N-methylmorpholine; DMF, dimethylformamide; DIPEA, *N*,*N*-diisopropylethylamine; DCM, dichloromethane

* Corresponding authors at: Universidade Federal de São Paulo (UNIFESP), Department of Biophysics, Rua Pedro de Toledo, 669, Enzymology Laboratory – 7th Floor, São Paulo, Brazil. Tel./fax: +55 11 55764450x1966 (V. Oliveira). Universidade Federal de São Paulo (UNIFESP), Department of Biophysics, Rua 3 de maio, 100, Ed INFAR 2nd Floor, São Paulo, Brazil. Tel./fax: +55 11 55764450x1960 (M.A. Juliano).

E-mail addresses: vitor.oliveira@unifesp.br (V. Oliveira), ma.juliano@unifesp.br (M.A. Juliano).

[1] Current address: Bruker do Brasil, Atibaia, SP, Brazil.

http://dx.doi.org/10.1016/j.fob.2015.05.004
2211-5463/

oct1 processed proteins in a Δoct1 *S. cerevisiae* [14]. Thus, oct1 converts unstable precursor intermediates generated by MPP into stable mature proteins. This same function of stabilizing proteins, because of removal of a N-terminal residue in proteins imported to mitochondrial matrix, has also been proposed for the recently identified amino peptidase lcp55 [15]. Using proteomic approaches directed to yeast mitochondrial N-terminal sequences, the authors showed that this amino peptidase also acts after MPP processing, by removing one amino acid residue from the newly generated N-terminal. Therefore, some proteins are processed in two steps: the first by MPP followed by oct1 or first by MPP but followed by lcp55. There is also the possibility that some proteins undergo a three step processing MPP-lcp55-oct1, or MPP-oct1-oct1 [13,15].

using the fluorogenic substrate Abz-GFSPFRQ-EDDnp [16]. Only a small level of inhibition was detected with thiorphan (a neprilysin inhibitor) [17] captopril (an angiotensin-converting enzyme inhibitor) [18] and JA-2 (a thimet oligopeptidase inhibitor) [19]. These results exclude *h*MIP as a target of these inhibitors that are used widely to measure TOP (thimet oligopeptidase), ACE (angiotensin-converting enzyme) or NEP (neprilysin) enzymatic activity in cells, tissue sample extracts, and also for *in vivo* inhibition assays. These metallopeptidase inhibitors bear a substrate mimetic portion containing a zinc binding group as warhead. A good understanding of the substrate specificity of *h*MIP can guide the design of new selective inhibitors containing this zinc binding group as warhead.

Table 1
Substrate preference matrix for *h*MIP.

	P_6	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	P_6'
Ala			1	1	1	2	1	1	2	2	3	1
Leu			1		1			1	4	1		
Ile			1		1		2	2	1	4	1	1
Val				2	2	1	1	3	3	2	1	
Phe	1				1							
Tyr												2
Trp					1							
Ser				1		5	8	2				
Thr				1		4	6	4	2	3	3	1
Asn		1				1						
Gln					2	5	2	1				
Asp		1										
Glu					1							
Lys				1	2	1		3	4	2	1	1
Arg								1		1		
His												
Gly								1	2			
Pro			1									
Met				1		1				1		
Cys												
	1	2	4	7	12	20	20	19	18	16	9	6

Analysis of the frequencies for specific residues at each identified position in the sequences of peptides hydrolysed in the support-bound FRET peptide library screening. Based on the 20 sequences shown in Table S1. The bottom rows indicate the total number of residues analyzed for each specific position. The background grey scale helps to bring attention to the higher numbers.

We recently reported the expression, purification and the partial characterization of recombinant human MIP in *Escherichia coli*, and presented the first continuous activity assay for this peptidase,

Knowledge about the substrate specificity of peptidases is essential to identify their roles in mammalian organisms and to aid in the development of selective assays. FRET substrates are