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

M.F.M. Marcondes, F.M. Alves, D.M. Assis, 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 – 7th andar, São Paulo, Brazil

Abstract

The substrate specificity of recombinant human mitochondrial intermediate peptidase (hMIP) using a synthetic support-bound FRET peptide library is presented. The collected fluorescent beads, which contained the hydrolysed peptides generated by hMIP, were sequenced by Edman degradation. The results showed that this peptidase presents a remarkable preference for polar uncharged residues at P1 and P3 substrate positions: Ser > Gln > Thr at P1 and Ser > Thr at P3. Non-polar residues were frequent at the substrate P8, P6, P5' and P4' 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 P6 and a polar uncharged residue (Ser or Thr) at P8. 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 P1 and P3 substrate positions for the hydrolytic activity of hMIP. The information presented in this work will help in the design of new substrate-based inhibitors for this peptidase.

© 2015 The Authors. Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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-MPP-oct1 [13,15].

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, 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 hMIP 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 hMIP can guide the design of new selective inhibitors containing this zinc binding group as warhead.

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

| Table 1 | Substrate preference matrix for hMIP. |
|--------|-------------------------------------|
|        | P₈ | P₇ | P₆ | P₅ | P₄ | P₃ | P₂ | P₁ | P₀ | P₋₁ | P₋₂ | P₋₃ | P₋₄ | P₋₅ | P₋₆ |
| Ala    | 1  | 1  | 1  | 2  | 1  | 2  | 2  | 3  | 1  |     |     |     |     |     |     |
| Leu    | 1  | 1  | 1  | 2  | 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    | 1  |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Trp    | 1  |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Ser    | 1  | 3  | 8  | 2  |     |     |     |     |     |     |     |     |     |     |
| Thr    | 1  | 4  | 6  | 4  | 2  | 3  | 1  |     |     |     |     |     |     |     |
| Asn    | 1  |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Gln    | 1  | 2  | 5  | 2  | 1  |     |     |     |     |     |     |     |     |     |
| Asp    | 1  |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Glu    | 1  |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Lys    | 1  | 2  | 1  | 3  | 4  | 2  | 1  | 1  |     |     |     |     |     |     |
| Arg    | 1  |     |     |     |     |     |     |     |     |     |     |     |     |     |
| His    | 1  |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Gly    | 2  | 1  |     |     |     |     |     |     |     |     |     |     |     |     |
| Pro    | 1  |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Met    | 1  |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Cys    | 1  | 4  | 7  | 12 | 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.
Table 2
Kinetic parameters for the hydrolysis of FRET peptides by hMIP.

| Sequence No. | Substrate                        | $k_{cat}$ (s$^{-1}$) | $K_M$ (mM) | $k_{cat}/K_M$ (mM$^{-1}$ s$^{-1}$) |
|-------------|----------------------------------|----------------------|------------|-----------------------------------|
| 1           | Abz-VAA(QTKTK(Dnp)-NH$_2$)       | 0.050                | 10         | 5.0                               |
| 2           | Abz-WT[GKKK(Dnp)-NH$_2$]         | 0.0033               | 6.2        | 0.53                              |
| 4           | Abz-MT[LALK(TK(Dnp)-NH$_2$)     | 0.19$^a$             | 4.2$^a$    | 45$^a$                            |
| 5           | Abz-[RQQ]SSLKK(Dnp)-NH$_2$       | 0.055                | 1.1        | 50                                |
| 6           | Abz-NLM[KKKSTK(Dnp)-NH$_2$      | 0.028                | 1.3        | 22                                |
| 8           | Abz-[FS]SCTR[TK(Dnp)-NH$_2$]    | 0.036                | 1.3        | 28                                |
| 10          | Abz-[IS]SRLEK(Dnp)-NH$_2$        | 0.083                | 1.9        | 44                                |
| 12          | Abz-TTKL; K; A[AK(Dnp)-NH$_2$    | 0.070$^a$            | 0.85$^a$   | 82$^a$                            |
| 14          | Abz-FQ[TKVAAK(Dnp)-NH$_2$       | 0.057                | 3.4        | 17                                |

The substrates were synthesized based on the sequences identified in the support-bound FRET peptide library screening. The parameters were calculated as mean value ± SD, which was lower than 10%. $^a$ indicates the cleavage site.

Table 3
Substrate preference matrix for hMIP.

| $P_5$ | $P_6$ | $P_7$ | $P_8$ | $P_9$ | $P_{10}$ | $P_{11}$ | $P_{12}$ | $P_{13}$ | $P_{14}$ | $P_{15}$ | $P_{16}$ |
|-------|-------|-------|-------|-------|----------|----------|----------|----------|----------|----------|----------|
| Ala   | 1     | 5     | 3     | 3     | 3         | 4         | 2         | 2         |          |          |          |
| Leu   | 6     |       |       |       |           |           |           |           |          |          |          |
| Ile   | 2     |       | 1     |       |           |           |           |           |          |          |          |
| Val   | 1     | 2     | 6     | 1     | 4         | 1         | 9         | 6         | 3         |          |          |
| Phe   | 10    |       |       |       |           |           |           |           |          |          |          |
| Tyr   | 1     |       |       |       |           |           |           |           |          |          |          |
| Trp   | 1     | 1     |       |       |           |           |           |           |          |          |          |
| Ser   | 8     | 2     | 13    | 1     | 1         | 4         | 5         | 5         | 2         | 3         | 2        |
| Thr   | 1     | 8     | 3     | 1     | 1         | 1         | 1         | 1         |          |          |          |
| Asn   |       | 1     | 2     | 2     | 2         | 1         |          |          |          |          |          |
| Gln   | 2     | 1     |       |       |           |           |           |           |          |          |          |
| Asp   |       | 2     |       |       |           |           |           |           |          |          |          |
| Glu   |       |       |       |       |           |           |           |           |          | 1         | 2        |
| Lys   | 3     |       |       |       |           |           |           |           | 5         | 1         | 1        |
| Arg   | 3     |       |       |       |           |           |           |           | 1         | 2         | 1        |
| His   | 2     |       |       |       |           |           |           |           | 2         |          |          |
| Gly   | 5     |       |       |       |           |           |           |           | 1         | 1         | 1        |
| Pro   | 4     |       |       |       |           |           |           |           | 1         |          |          |
| Met   |       |       |       |       |           |           |           |           | 1         |          |          |
| Cys   | 1     | 2     | 2     |       |           |           |           |           |           |          |          |

Analysis of the frequencies for specific residues, at each position, in the sequences of predicted MIP processing sites at the newly generated N-terminal by the MPP action on nuclear encoded proteins imported to the mitochondrial matrix [25]. Based on the 19 sequences shown in Table S3. The background grey scale helps to bring attention to the higher numbers.
useful tools to evaluate the substrate specificity of MIP and an important step for the development of specific inhibitors. Because MIP activity contributes to the mitochondrial iron uptake it has been proposed that the inhibition of MIP might decrease the mitochondrial iron accumulation, a disease known as Friedreich's ataxia, and this could attenuate some complications of this neurodegenerative disease [20].

In the present work, we studied the substrate specificity of recombinant human MIP (hMIP), using the synthetic support bond FRET peptide library approach. This support-bound FRET peptide library was prepared on polyethylene glycol dimethyl acrylamide copolymer (PEGA) beaded resin by the process of split-combine synthesis, which results in a single peptide sequence on each bead [21–24]. Seven positions (X1–X7) were randomized using all 20 genetically encoded amino acids. FRET peptides when hydrolysed by the peptidase turned the bead fluorescent and these beads were manually picked, the remaining peptide was sequenced by Edman degradation methodology. In this approach the enzymatic reaction is terminated before complete hydrolysis of all the peptide attached to a single bead, so each bead contained both the complete peptide sequence as well as the sequences of the remaining portion of the cleaved peptide (S amino acids). Thus, is possible to obtain the peptide sequence and the cleavage site using this experimental approach.

9 identified peptides using this approach were synthesized, the hMIP cleavage sites and the kinetic parameters evaluated. Additional kinetic analyses were also carried out, using synthetic fluorogenic substrates based on the processing sites attributed to MIP for the precursor nuclear encoded proteins. The data of these substrates gave further insights into the hMIP substrate specificity.

2. Results and discussion

2.1. Support-bound FRET peptide library screening

The results of the Edman degradation analysis of the fluorescent beads collected in the synthetic support-bound FRET peptide library screening with hMIP are shown in Table S1. The results of this assay clearly showed that MIP hydrolysed preferentially the peptides presenting uncharged polar residues at both P1 and P1′ positions, especially Ser, Thr and Gln (Tables S1, S2 and Table 1). Thr and Ser are also the preferred residues found in the P2′ position (Table 1). Beyond the clear preference for uncharged polar residues at positions P1, P1′ and P3′, some other determinants of specificity were also observed for hMIP in the assays: hMIP prefers nonpolar residues at the P3, P2, and P3′ substrate positions (Table 1). However, the occurrence of the basic residue Lys at position P2′ and P3′ is significant (Table 1).

Fig. 1. 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 in comparison with the hydrolysis of the FRET substrates in the solution, and the sequences based on proteins with confirmed two-step cleavage by MIP [25].
Based on the results obtained in the support-bound FRET peptide library screening 9 new FRET substrates were synthesized. The sequences are shown in Table 2, these FRET substrates were assayed in solution with hMIP, and the kinetic parameters for the hydrolysis reactions are presented in Table 2. All substrates were hydrolysed by hMIP; 5 of them at the same site as it occurred in the solid-phase support, but 4 were cleaved at a different site or presented additional cleavages by MIP. However, despite these differences between the cleavage sites observed after the kinetic assays in the FRET substrates and the cleavage sites detected in the support bound peptide only the substrates 6 and 12 were not hydrolysed with uncharged polar residues at P1 or P1′.

For the substrates 4 and 12 more than one cleavage site were detected (Table 2), analysis of product formation in time using HPLC revealed that for the both substrates the peaks corresponding to the products appears simultaneously indicating that no subsequent cleavage of the products occurs. These data together with a description that MIP can act on some FRET substrates described for the thimet oligopeptidase (TOP) and neurolysin (NEL) [16], oligopeptidases that are members of MIP peptidase family, shows that MIP is able to act on peptides releasing products with different lengths, not only 8-mer peptides. A possible explanation for those observations is that when acting on the N-terminal of large proteins MIP probably presents some ruler-like interaction but when small substrates are free to find their way into the MIP active site fewer interactions seems to be necessary to the formation of a productive enzyme-substrate complex. Unfortunately this characteristic of MIP cannot be interpreted in details, since the 3D structure of MIP has not been determined so far.

Therefore, also in the case of these small substrates in solution, which are free to access the hMIP extended substrate binding site with multiple possibilities of interaction it can be observed that uncharged polar residues are preferred at P1 and/or P1′ substrate positions (Table 2).

Table 4
Substrate preference matrix for oct1.

|     | Pₐ | P₇ | P₆ | P₅ | P₄ | P₃ | P₂ | P₁ | P₁’ | P₂’ | P₃’ | P₄’ | P₅’ |
|-----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Ala | 1  | 1  | 1  | 3  | 1  | 1  | 1  | 2  | 2  | 1  | 1  |     |     |
| Leu | 2  | 1  | 2  | 4  | 1  |    |    |    |    |    |    |     |     |
| Ile | 1  | 1  | 1  | 1  |    |    |    |    |    |    |    |     |     |
| Val | 1  | 2  | 1  | 3  | 1  |    |    |    |    |    |    | 2   |     |
| Phe | 11 | 1  | 1  | 1  | 1  |    |    |    |    |    |    |     |     |
| Tyr | 1  | 1  | 1  | 1  | 1  |    |    |    |    |    |    |     |     |
| Trp | 1  | 1  | 1  | 1  | 1  | 1  |    |    |    |    |    |     |     |
| Ser | 5  | 6  | 6  | 1  | 1  | 2  | 1  | 2  | 1  | 1  | 1  | 2   | 2   |
| Thr | 3  | 3  | 4  | 2  | 2  | 1  | 1  | 1  | 1  | 1  | 1  |     |     |
| Asn | 1  | 1  | 2  | 1  | 2  | 1  | 1  | 1  | 1  | 1  | 1  |     |     |
| Gin | 2  | 4  | 4  | 1  | 1  | 1  |     |     |     |     |     |     |     |
| Asp | 1  | 1  | 1  | 1  | 1  |     |     |     |     |     |     |     | 3   |
| Glu |     | 1  | 1  |     |     |     |     |     |     |     |     |     |     |
| Lys | 2  | 1  | 1  | 2  | 3  | 4  | 3  | 1  |     |     |     |     |     |
| Arg | 1  | 2  | 1  | 1  | 2  | 1  | 1  | 1  |     |     |     |     |     |
| His | 2  | 2  | 1  | 1  | 1  | 1  | 1  | 1  |     |     |     |     |     |
| Gly |     | 2  | 2  |     |     |     |     |     |     |     |     |     |     |
| Pro | 2  | 1  | 1  | 1  | 1  | 1  | 1  |     |     |     |     |     |     |
| Met | 1  | 1  | 1  | 1  | 1  | 1  |     |     |     |     |     |     |     |
| Cys | 1  | 2  | 1  |     |     |     |     |     |     |     |     |     |     |

Analysis of the frequencies for specific residues at each position in the sequences of predicted oct1 processing sites at the newly generated N-terminal by the MPP action on nuclear encoded proteins imported to the S. cerevisiae mitochondrial matrix [14]. Based on the 14 sequences shown in Table S4. The background grey scale helps to bring attention to the higher numbers.
2.2. Specificity analysis based on natural substrates

Joseph et al. analyse the sequences of proteins known as substrates for MIP and identified some conserved positions [25], with respect to the cleaved bond. These conserved positions are F/I/L at P₈ and T/S/G at P₉. Reanalysing both predicted and confirmed MIP cleavage sites on 19 precursor proteins, we noted that 14 (>70%) of them have polar uncharged residues at P₁ and/or the P₁' positions [25] (Table 3 and Table S3), results in line with the outcome of the support-bound FRET peptide library presented above (Table 1).

The substrate binding preference of the P₃–P₉' sites of MIP are presented in the Fig. 1, based on the data obtained from the synthetic support-bound FRET peptide library, the hydrolysis of the 9 small FRET substrate in solution, and the sequences of precursor proteins that have confirmed the second step of cleavage by MIP [25] (Table S3). This analysis corroborates the preference for non-polar residues at the P₂ position; the occurrence of nonpolar or basic residues at the P₂', detected in the support-bound FRET peptide analysis, and the clear preference for uncharged polar residues at positions P₁ and P₁' (Fig. 1).

Recently MIP processing sites were studied in details for yeast mitochondria imported proteins [14]. This study revealed important observations about the yeast MIP – the so-called oct1 – to light. Table 4 shows the MPP processing sites together with the MIP processing sites in 14 mitochondrial yeast proteins. Again F/I/L at the position –8 and T/S/G at –5 seem to be determinants to MPP/MIP activities (Table 4 and Table S4). Furthermore, Isaya et al. described additional evidences that hydrophobic amino acid positioned near the N-terminal residues are crucial for MIP activity [26].

The results described here support the knowledge that MIP can act on small FRET substrates [16] and the importance for the P₁ and P₁' substrate residues, close to the hydrolysed peptide bond. However the impact of this F/I/L at P₈ and T/S/G at P₉ positions on the ability of MIP to process small peptides its unknown. To investigate this, we synthesized 2 FRET substrates containing the sequences of precursor proteins – assumed to be hydrolysed by MIP – mimicking the predicted processing sites, Abz-FRSQGPLQKVKQLQ-EDDnp (human ornithine transcarbamylase) and Abz-VRTLTINK_EDDnp (yeast pyruvate dehydrogenase E3 subunit). Both substrates were cleaved at the same predicted site, Abz-FRSQGPLQKVKQLQ-EDDnp and Abz-VRTLTINK_EDDnp, however in the yeast sequence there is no P₈ nor Thr or Ser at P₉. The substrate Abz-FRSQGPLQKVKQLQ-EDDnp is the best substrate for hMIP with better specificity constant due to the higher catalytic constant value (kₐₛ /Kₐₘ = 130 mM⁻¹ s⁻¹ and kₐₛ = 0.122 - s⁻¹), when compared with kinetic parameters of Abz-VRTLTINK_EDDnp (kₐₛ /Kₐₘ = 27 and kₐₛ = 0.0093). No significant difference on the affinity constant values (Kₐₘ) was observed, 0.941 mM⁻¹ and 0.35 mM⁻¹ respectively.

Based on this observation, some variations of the human ornithine transcarbamylase based substrate, Abz-FRSQGPLQKVKQLQ-EDDnp, were prepared and assayed with hMIP. These variants were synthesized with the purpose to analyse the importance of the substrate length, especially the impact of both P₈ and P₉ positions, and also the importance of the residues at P₁ and/or P₁' positions. Furthermore, these new variants could be an interesting starting point for the development of new specific FRET substrates for MIP activity detection assays. The data of these new substrates, the results of cleavage site and kinetic parameters are presented in Table 5. The kinetic assays with the variants of the Abz-FRSQGPLQKVKQLQ-EDDnp gave all similar results. The smaller variants Abz-QPLQKVKQLQ-EDDnp, Abz-QPLANKVQ-EDDnp and Abz-QPLQAKVKQ-EDDnp, were all cleaved at the same site. However, the analogue Abz-QPLQKVKQ-EDDnp, without the P₈, was hydrolysed with a 7-fold lower specificity constant than Abz-FRSQGPLQKVKQLQ-EDDnp (Table 5).

3. Conclusions

The hMIP substrate specificity was first investigated using synthetic support-bound FRET peptide library screening, the results obtained in this entire randomized approach revealed the hMIP preferences for substrate residues close to the hydrolysed peptide bond, with a clear preference for uncharged polar residues at positions P₁ and P₁' and nonpolar residues at the substrate P₈, P₉, P₁' and P₁' positions, matching exactly the analysis of the cleavage sites predicted to be natural substrates for hMIP.

The results of the specificity analysis using FRET substrates based on natural substrates, support the information that hMIP can act upon small substrates, as well as that for recognition and processing small peptides the most important conserved position (F/I/L at P₈ and T/S/G at P₉) are not crucial, as in the case of the action by hMIP on the N-terminal of large proteins. This observation together with the distinct substrate specificity presented by hMIP, specially its preference for polar uncharged residues at P₁ and P₁' positions, can be important information for the development of substrates and inhibitors specific for hMIP.

4. Experimental procedures

4.1. Construction of the expression vector

The hMIP cDNA fragment (~2000 bp) was amplified by PCR using the clone, ID 4053687, from the Mammalian Gene Collection (MGC) of Invitrogen. The construct pET28-MIP, which produces hMIP with a hexahistidine tag at its N-terminus instead of the residues that correspond to the mitochondrial signal sequence, has already been previously described by our group [16].

4.2. Expression of recombinant hMIP

E. coli BL21 (DE3) pLysS (Novagen) was used as the expression host. E. coli cells were incubated overnight at 30 °C and shaken at 150 rpm in 10 mL of Luria-broth (LB) medium, containing kanamycin (50 µg/mL) and chloramphenicol (50 µg/mL). These cells were transferred to 1 L of fresh LB medium at 30 °C and 150 rpm, until the culture density reached 0.4 OD₅₅₀. The temperature was then reduced to 20 °C and when the culture density reached 0.6–0.7 OD₅₅₀ isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. After 14–16 h, the bacterial cells

Table 5 Kinetic parameters for the hydrolysis of FRET peptides by hMIP.

| Substrate                          | kₛ (s⁻¹) | Kₐₘ (µM) | kₛ/Kₐₘ (mM⁻¹ s⁻¹) |
|------------------------------------|----------|----------|-------------------|
| Abz-FRSQGPLQKVKQLQ-EDDnp           | 0.223    | 1.80     | 124               |
| Abz-FRSQGPLQKVKQLQ-EDDnp           | 0.122    | 0.941    | 130               |
| Abz-QPLQKVKQ-EDDnp                 | 0.061    | 2.9      | 21                |
| Abz-QPLANKVQ-EDDnp                 | 0.0570   | 5.1      | 11                |
| Abz-QPLQAKVKQ-EDDnp                | 0.0943   | 3.2      | 29                |

The parameters were calculated as mean value ± SD, which was lower than 10%, ↓ indicates the cleavage site.
were harvested by centrifugation at 5000 rpm, for 10 min, at 4 °C. The pellet was stored at −70 °C.

4.3. Purification of the recombinant hMIP

The pellet was resuspended in 20 mL binding buffer (50 mM NaH2PO4, 500 mM NaCl, 20 mM imidazole, pH 8.0), and lysozyme (1 mg/mL). This suspension was put on ice for 30 min and then RNase and DNase (to a final concentration of 5 μg/mL each), and 2 mL of 0.2% Triton X-100 were added. The resultant mixture was centrifuged at 15,000 rpm for 20 min and the supernatant was recovered.

The supernatant was loaded at a flow rate of 0.5 mL/min on a Ni-Sepharose high performance chromatography column (GE Healthcare) previously equilibrated with binding buffer. The column was washed with 5 mL of binding buffer, and the recombinant hMIP was eluted, using a segmented step elution with increasing imidazole concentrations (50, 100, 150 and 500 mM) in binding buffer (20 mM NaH2PO4, pH 8.0, 500 mM NaCl). The recombinant protein eluted between 100 and 150 mM imidazole. Fractions of 8–10 mL each, were collected and loaded onto a desalting preparatory column (GE Healthcare), and the fractions containing hMIP were recovered.

This desalted samples were loaded at a flow rate of 1.0 mL/min on a resource Q column (1 mL) previously equilibrated with TB buffer (50 mM Tris, pH 7.4). After an initial washing with 6 mL of TB buffer, the elution was performed with 40 mL of a linear gradient with TBS buffer (50 mM Tris–HCl, pH 7.4, 500 mM NaCl). Recombinant hMIP eluted between 80 and 200 mM NaCl. The fractions containing homogeneous (SDS–PAGE) recombinant hMIP, according to SDS PAGE analysis, were concentrated using an Amicon filtration unit (Millipore Corp.) equipped with a 50 kDa exclusion membrane, and the recovered protein was finally stored in TBS buffer at −70 °C.

4.4. Peptide synthesis

Highly sensitive FRET peptides were synthesized by solid-phase procedures, as described elsewhere [27].

4.5. Synthesis of support-bound FRET peptide library

The syntheses of libraries were carried out manually as previously described [24]. Briefly, the libraries were synthesized using 1 g of PEGA 1900 resin [28] in a 20 column Teflon synthesis block, using protected Fmoc amino acids. The resin was evenly distributed in the 20 wells of the Teflon synthesis block, and Fmoc groups were removed. Prior to coupling, the Fmoc amino acids (1 equiv.) were pre-activated with HOBt (1 equiv.), TBTU (1 equiv.) and NMM (2 equiv.) in DMF (1 mL) for 6 min; on the activated amino acids were added to each of the 20 wells. After the completion of the coupling, the block was filled with DMF up to 1 cm above the top of the wells and inverted. Then, the resin was mixed vigorously by agitation for 30 min in the mixing chamber. The block was again inverted, evenly distributing the resin in the wells for washing and removal of Fmoc group. This procedure was repeated for the incorporation of all the randomized positions. After the randomized positions, the Fmoc-K (Abz-Boc) and Fmoc-K (Dnp) were incorporated. The side chain protecting groups were removed by treatment with a mixture of TFA:thioanisole:ethane dithiol:water (87:5:5:3) for 8 h. The resin was washed with 95% acetic acid (4×), DMF (4×), 5%DIPEA in DMF (3×), DMF (3×), DCM (6×) and finally dried under vacuum.

4.6. Support-bound FRET peptide library screening

The peptide library screening was carried out as previously described [24]. For all assays, the library beads were washed with water (3×) and the assay buffer (3×) before the addition of the enzyme. The reactions were stopped by dilution with 3 M HCl, and the mixtures were washed thoroughly until pH 5.6 was reached. The beads were transferred to a glass dish and inspected by fluorescence microscopy (Stereo microscope Stemi-Zeiss), and the fluorescent beads were collected and transferred to a TFA-treated cartridge filter for on-resin sequence analysis. The amino acid sequence and cleavage site were determined by Edman degradation using a PPSQ/23 protein sequencer (Shimadzu, Japan). hMIP was assayed as follows: 0.5 μM of enzyme was mixed with 50 mg of resin 50 mM Tris, 100 mM NaCl, pH 8.0, at 25 °C for 24 h. The identified peptide sequences susceptible to hydrolysis by hMIP were synthesized as FRET Abz-peptidyl-Q-EDDnp peptides and assayed in solution with the enzyme.

4.7. Enzymatic activity assay

Hydrolysis of the FRET peptides used as substrates for hMIP was monitored spectrophotometrically in a Shimadzu RF-5301PC spectrophotometer with excitation and emission wavelengths of 320 and 420 nm, respectively. The rate of increase in fluorescence was converted into moles of substrate hydrolysed per second, based on the fluorescence curves of standard peptide solutions, before and after total enzymatic hydrolysis. The enzyme concentration for initial rate determinations was chosen so that <10% of the substrate was hydrolysed. The activity was measured in the absence or in the presence of the assayed inhibitors [16].

4.8. Determination of peptide cleavage sites

The peptide cleavage sites were identified using mass spectrometry (LCMS-2010 EV Shimadzu, Tokyo, Japan) and/or peptide sequencing, using a PPSQ-23 protein sequencer (Shimadzu, Tokyo, Japan) [16].

Author’s contributions statement

MMFM – Planned experiments, performed experiments, analyzed data, wrote the paper; FMA – Planned experiments, performed experiments, analyzed data; DMA – Performed experiments, analyzed data; IYH – Performed experiments, analyzed data; VO – Planned experiments, analyzed data, wrote the paper; MAJ – Planned experiments, performed experiments, analyzed data, wrote the paper.

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – Projects: 12/50191-4R; 471340/2011-1; 470388/2010-2; 306587/2010-6).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2015.05.004.
References

[1] van der Laan, M., Rissler, M. and Rehling, P. (2006) Mitochondrial preprotein translocases as dynamic molecular machines. FEMS Yeast Res. 6, 849–861.

[2] Gakh, O., Cavadin, P. and Isaya, G. (2002) Mitochondrial processing peptides. Biochim. Biophys. Acta 1592, 63–77.

[3] Gavel, Y. and von Heijne, G. (1990) Cleavage-site motifs in mitochondrial targeting peptides. Protein Eng. 4, 33–37.

[4] Isaya, G., Kalousek, F., Fenton, W.A. and Rosenberg, L.E. (1991) Cleavage of precursors by the mitochondrial processing peptide requires a compatible mature protein or an intermediate octapeptide. J. Cell Biol. 113, 65–76.

[5] Kalousek, F., Hendrick, J.P. and Rosenberg, L.E. (1988) Two mitochondrial matrix proteases act sequentially in the processing of mammalian matrix enzymes. Proc. Natl. Acad. Sci. U.S.A. 85, 7536–7540.

[6] Brand, S.S. and Isaya, G. (1995) Prediction and identification of new natural substrates of the yeast mitochondrial intermediate peptidase. J. Biol. Chem. 270, 27366–27372.

[7] Chew, A., Rollins, R.A., Sakati, W.R. and Isaya, G. (1996) Mutations in a putative zinc-binding domain inactivate the mitochondrial intermediate peptidase. Biochem. Biophys. Res. Commun. 226, 822–829.

[8] Chew, A., Sirugo, G., Alsbobrook 2nd, J.P. and Isaya, G. (2000) Functional and genomic analysis of the human mitochondrial intermediate peptidase, a putative protein partner of frataxin. Genomics 65, 104–112.

[9] Isaya, G., Kalousek, F., Hendrick, J.P. and Rosenberg, L.E. (1991) Cloning, expression, and chromosomal assignment of the human mitochondrial intermediate peptidase gene (MIPEP). Genomics 10, 493–496.

[10] Isaya, G., Mikielos, D. and Rollins, R.A. (1994) MiPI, a new yeast gene homologous to the rat mitochondrial intermediate peptidase gene, is required for oxidative metabolism in Saccharomyces cerevisiae. Mol. Cell. Biol. 14, 5603–5616.

[11] Isaya, G., Sakati, W.R., Rollins, R.A., Shen, G.P., Hanson, L.C., Ullrich, R.C. and Novotny, C.P. (1995) Mammalian mitochondrial intermediate peptidase: structure/function analysis of a new homologue from Schizopyllum commune and relationship to thimet oligopeptidases. Genomics 28, 450–461.

[12] Branda, S.S., Yang, Z.Y., Chew, A. and Isaya, G. (1999) Mitochondrial intermediate peptidase and the yeast frataxin homolog together maintain mitochondrial iron homeostasis in Saccharomyces cerevisiae. Hum. Mol. Genet. 8, 1099–1110.

[13] Hendrick, J.P., Hodges, P.E. and Rosenberg, L.E. (1989) Survey of amino-terminal proteolytic cleavage sites in mitochondrial precursor proteins: leader peptides cleaved by two matrix proteases share a three-amino acid motif. Proc. Natl. Acad. Sci. U.S.A. 86, 4056–4060.

[14] Alves, F.M., Hirata, I.Y., Boschcov, P., Oliveira, M.C., Juliano, M.A., Miranda, A., Chagas, J.R., Tsboi, S., Okada, Y. and Juliano, L. (1991) Synthesis of human angiotensinogen (1–17) containing one of the putative glycosylation binding sites and its hydrolysis by human renin and porcine pepsin. Int. J. Pept. Protein Res. 38, 298–307.

[15] Booze, M., Christensen, S.F. and Meldal, M. (2004) ULTRAMINE: a high-capacity polyethylene-imine-based polymer and its application as a scavenger resin. Chem. 10, 4407–4415.