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Substrate evokes translocation of both domains in the mitochondrial processing peptidase α-subunit during which the C-terminus acts as a stabilizing element

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Abstract

All three tryptophan residues in α-subunit of mitochondrial processing peptidase (MPP) were subsequently substituted. While substitutions of Trp^{223} led to misfolded non-functional protein, mutations of Trp^{147} and/or Trp^{481} did not affect the enzyme processing activity. Thus, fluorescence properties of the mutants with fewer tryptophans were used for observation of both α-MPP domain translocation and visualization of conformational changes in the interdomain linker evoked by substrate. We found that in the presence of substrate the C-terminal penultimate Trp^{481} was approaching Trp^{223}, which is localized at the border of N-terminal domain and interdomain linker. Also, excision of the α-MPP C-terminal 30 amino acid residues (ΔC30) led to a complete loss of protein function. Even shorter deletions of the α-MPP C-terminus destabilized the protein slightly (ΔC2) or dramatically (ΔC17). It suggests that the extreme C-terminus of α-MPP provides mechanical support to the C-terminal domain during its extensive conformational change accompanying the substrate recognition process.

Keywords: Metallopeptidase; Mitochondria; Mitochondrial processing peptidase; Presequence; Processing; Substrate recognition; Tryptophan fluorescence measurement

Mitochondrial processing peptidase (MPP; EC 3.4.24.64) is a metallopeptidase that cleaves off most of the N-terminal presequences from precursor proteins imported into the mitochondria [1–3]. Those presequences vary in length and sequence [2,4] and adopt context-dependent conformations through mitochondrial import and processing [5]. Mitochondrial import machinery recognizes amphiphilic helical conformations in signal sequences [6]. Yet, crystal structures of two different synthetic substrate peptides cocrystallized with mutant MPP deficient in cleavage function show the peptide bound in an extended conformation at the active site [7].

Both MPP homologous subunits, α and β, are essential for its processing activity. The catalytic center is conserved only in the β-subunit. Crystal structure of yeast MPP [7] demonstrates the nearly identical protein architecture of the subunits. Each subunit consists of two domains of ~210 residues with similar folding topology connected by a flexible linker of ~20 residues. Thus, the MPP dimer consists of four structurally related domains.

A striking feature of MPP is its acting on various mitochondrial precursor proteins, while being specific as it recognizes a distinct cleavage site on unlike presequences. Apparently, there are conflicting results

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concerning the substrate recognition process by MPP. On one hand, it is known, both MPP subunits cooperatively control translocation of the substrate presequences to the polar internal cavity inside of the enzyme dimer and to the proximity of the active site in β-MPP. Enzyme bound the substrate peptides with high affinity only in the dimeric complex [8]. On the other hand, it remains unclear which subunit and their particular regions are responsible for the primary substrate–enzyme interaction as well as the mechanism of substrate entrance into a polar cavity of the enzyme. Several results indicated the importance of α-MPP. First, mutations in the glycine-rich segment, the highly conservative region of all α-MPPs, exposed to the catalytic center of the β-MPP as well as to the substrate, led to a dramatic reduction in processing activity practically to zero [9]. Second, truncation of the α-MPP C-terminal 41 amino acid residues led to a loss of binding and processing activity [10]. Third, cross-linking and surface plasmon resonance analyses showed that α-MPP, but not β-MPP, binds substrates as efficiently as does the MPP complex [11,12]. Finally, lifetime analysis of tryptophan fluorescence of the yeast MPP subunits proved that substrate binding evoked a conformational change of α-MPP, but not β-MPP [13].

There are three tryptophan residues (Trp^{147}, Trp^{223}, and Trp^{481}) in the yeast α-MPP sequence. In order to determine more precisely the α-MPP region responsible for the conformational change detected previously, all three tryptophan residues were subsequently substituted. Systems with no tryptophan in the substrate and with one or two remaining Trp residues in α-MPP enabled us to monitor microenvironments of each individual tryptophan present in the native protein. In addition, we studied the function of the extreme C-terminus of yeast α-MPP in detail, as the data obtained from tryptophan fluorescence measurements indicated considerable complicity of the region in the overall subunit conformational change.

Materials and methods

Vectors. Constructs pETYA, pETYB, and pETPMDH were used for separate production of the mature forms of yeast α-MPP and β-MPP or the mouse malate dehydrogenase precursor (pMDH) as described previously [13,14]. DNA sequence coding for α-MPP from pETYA was transferred into pET28b(+) vector (Novagen, Madison, USA) to yield the pETYAH construct producing the N-terminally His-tagged yeast α-MPP.

Two primers, forward 5′ AACTGCAGCATATGTTGTCAAGA GTAGCTAAA 3′ (*Nde*I site underlined) and reverse 5′ GGAATTCT CGAGCTCTATTTCTGATCTTGAACA 3′ (*Xho*I site underlined), were used for amplification of the yeast full-length *mdh* sequence coding for the pMDH precursor. PCR was performed directly on the genomic DNA because no introns are present. The PCR product was treated with *Nde*I/*Xho*I and inserted into pET38b(+) (Novagen) to yield the pETyPMDH vector producing a homologous substrate of yeast MPP.

Site-directed mutagenesis of yeast α-MPP. Two types of mutations were performed in yeast α-MPP: (a) deletions of C-terminus and (b) substitutions of tryptophan residues. The deletion of the C-terminal 30 amino acid residues was performed by treatment of the pETYAH vector with *Bam*HI (site present naturally in the yeast α-MPP coding sequence) and *Xho*I (site originated from the pET28b vector polylinker sequence) and by insertion of compatible oligonucleotides (forward) 5′ GATCCTTT**TAG**TTAAC 3′ and (reverse) 5′ TCGAGTTAACTAAA A 3′, forming the translation termination codon (in bold). The deletion of 17 amino acid residues was performed analogically with a pair of longer oligonucleotides. For the deletion of the last two amino acid residues and for all tryptophan residue substitutions, a PCR-based approach was employed. Mutation primers carried the intended mutation. The construct producing α-MPP-W147N/W481Y was obtained by replacement of the *Hin*dIII–*Bam*HI fragment coding for W147N in the construct carrying the DNA sequence for α-MPP-W481Y. All mutants with substituted Trp residues were prepared with a His-Tag at the N-terminus, i.e., derived from the pETYAH construct.

Preparation of yeast MPP subunits and precursors in Escherichia coli. Heterologous proteins were separately produced in *E. coli* BL21(DE3) strain transformed with pETPMDH or pETyPMDH constructs for pMDH (mouse or yeast) precursor production or by pETYB for β-MPP expression. α-MPP was produced in *E. coli* BL21(DE3) co-transformed by pETYA or pETYAH with pGroESL (DuPont; vector bearing sequences coding for GroES and GroEL chaperonins). The mutant forms of α-MPP were produced accordingly using appropriate constructs. Transformed *E. coli* BL21(DE3) cells were grown on LB medium at 37 °C to $OD_{600} = 0.6$, induced with 0.4 mM IPTG, and cultivated for additional 2 h at 37 °C or for 36 h at 12 °C in case of α-MPP and derived mutant forms to assist proper protein folding [15]. Harvested cells were washed and sonically disrupted in HN buffer (20 mM Hepes–KOH, pH 7.4, 20 mM NaCl).

Purification of recombinant proteins. The isolation of the non-His-tagged wild type α-MPP or α-MPPΔC2 was carried out as by Geli [16] except that HN buffer was used. In the case of the ΔC17 deletion mutant, the cell-free extract was precipitated with ammonium sulfate to 35% saturation. The pellet was resuspended in HN buffer with 2% Triton, applied on a Q Sepharose Fast Flow column (Amersham–Pharmacia Biotech, Uppsala, Sweden), and eluted with a 0–500 mM NaCl gradient. N-terminally His-tagged α-MPP was isolated using a Hi-Trap Chelating column (Amersham, Little Chalfont, United Kingdom) equilibrated with TN buffer (20 mM Tris–HCl, pH 7.4, 100 mM NaCl, and 20% glycerol) and eluted (TN buffer; 250 mM imidazole). His-tags were cleaved off with thrombin (Invitrogen, Carlsbad, California, USA).

Insoluble β-MPP and both, yeast and mouse pMDH precursors, were isolated from inclusion bodies. The renaturation procedure was used as by Geli [17].

Evaluation of protein amount. The amount of the protein was evaluated after SDS–PAGE using the AIDA software (Advanced Image Data Analyzer 2.11; Raytest Izotopenmessgeraete, Straubenhardt, Germany).

Activity assay. To yield the active MPP dimer cell-free extract containing wild type or mutant α-MPP was mixed equimolarly with the β-subunit, incubated on ice for 30 min in the presence of 1 mM Mn^{2+}, and loaded on a 1 ml Hi-Trap Chelating column. The active MPP dimer was eluted (TN buffer, 250 mM imidazole) and incubated with mouse pMDH at 30 °C for 1, 5, 10, 20, and 30 min. In the case of α-MPPΔC30, impossible to purify, processing activity was determined directly in the cell-free extract containing the α-MPPΔC30 mixed equimolarly with the β-subunit.

Stability assay. Partial trypsin digestion was employed to characterize the stability of both the wild type α-MPP and the ΔC2 and ΔC17 mutant forms. The digestion was performed in an 80 μl reaction mixture (25 mM Tris, 25 mM KCl, and 10% glycerol) containing 10 μg α-MPP or the respective mutant, 10 ng trypsin, at 30 °C. Aliquots containing 1 μg of α-MPP were withdrawn at 0, 10, and 20 min and analyzed by SDS–PAGE.

Fluorescence measurement. Steady-state spectra of the tryptophan fluorescence were recorded on a Fluoromax-2 (Jobin-Yvox, Edison, New Jersey, USA) fluorometer at room temperature (22 °C). The excitation wavelength was 300 nm. To minimize the tyrosine fluorescence contribution, excitation and emission bandpasses were set to 3 nm. The α-MPP mutants were dissolved in HN buffer to a final concentration of 1 μM. The substrate was added in an equimolar concentration. The buffer and substrate (if present) signals were subtracted as background.

Distance r between Trp^{223} and Trp^{491} was calculated from the efficiency of Förster resonance energy transfer E as follows:

$$\frac{r_0^6}{r_0^6 + R_0^6} = 1 - E = \frac{F_{DA}}{F_D}, \quad (1)$$

where F_{DA} and F_D stand for the steady-state fluorescence intensities of donor in the presence or absence of the acceptor. R_0 is the Förster distance, at which the energy transfer efficiency is 50%. The quantum yield of the donor $\Phi_D = 0.32$ (in the presence of substrate) was estimated using D,L-tryptophan as a standard (QY = 0.14 [18]), the orientation factor κ^2 was taken as 2/3 assuming a random orientation of dipoles.

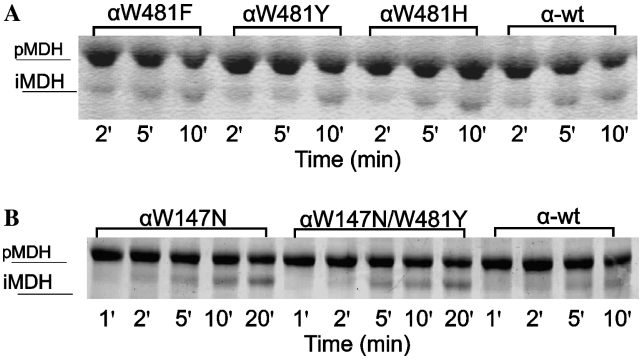

Fig. 1. Processing of pMDH by MPPs containing α-MPP with changed tryptophan residues. (A) Substitutions of Trp^{481}: W481F, W481Y, and W481H. (B) Mutations of Trp^{147}: W147N, W147N/W481Y. Precursor (pMDH) was incubated with 20 ng of the wild type or mutant MPPs at 30 °C for 2, 5, and 10 min (A) or 1, 2, 5, and 10 min (B) as described in Materials and methods. All presented forms of α-MPP were His-tagged. Processing products were analyzed on SDS–PAGE (10% gel stained with Coomassie blue). p, precursor form, i, intermediate form.

Results

Successive removal of tryptophan residues from yeast α-MPP

Tryptophan residues of yeast α-MPP (Trp^{147}, Trp^{223}, and Trp^{481}) were substituted separately to obtain an optimal model for tryptophan fluorescence assays used for monitoring the subunit–substrate interaction. Alignment performed on α-MPP sequences revealed that positions corresponding to the yeast α-MPP Trp residues are non-conserved. At the position corresponding to Trp^{147} of yeast α-MPP Trp, His, Ala, Thr, and twice Asn were found in sequences from *Neurospora*, *Blastocladiella*, *Arabidopsis*, potato, rat, and human, respectively. The substitution W147N was designed. Sequence alignments in regions of Trp^{223} (highly variable interdomain linker in the middle portion of the protein) or Trp^{481} (extreme C-terminus) are infeasible, regarding the sequence gaps. Thus, several conservative alternative substitutions were tested at both positions: W223M, W223F and W481Y, W481F or W481H. In addition, the deletion of C-terminal two residues Trp^{481}–Phe^{482} was prepared (α-MPPΔC2). None of the three alternative substitutions of the C-terminal penultimate residue Trp^{481} and the appropriate short deletion (ΔC2) affected the protein solubility. Nevertheless, the shortened α-MPPΔC2 tends to be slightly unstable during long-term storage as compared to substituted mutant forms of α-MPP. Similarly, the W147N substitution yielded fully soluble protein. Surprisingly, substitution of Trp^{223} led to the production of entirely (W223M) or nearly entirely (W223F) insoluble protein. The processing activities of all MPP dimers containing any soluble mutant forms of the α-subunit were equal to the wild type activity (Fig. 1). Consequently, we prepared a modified α-MPP with a single remaining Trp^{223} residue, i.e., α-MPP-W147N/W481Y. In that case, solubility of the protein and processing activity of the MPP dimer were comparable to those of the wild type (Fig. 1B).

Determination of the α-MPP subunit interaction with the precursor protein

Steady-state tryptophan fluorescence was measured to monitor conformational changes of α-MPP induced by the substrate binding. Emission spectrum of the mutant form containing a single Trp^{223} residue (α-MPP-W147N/

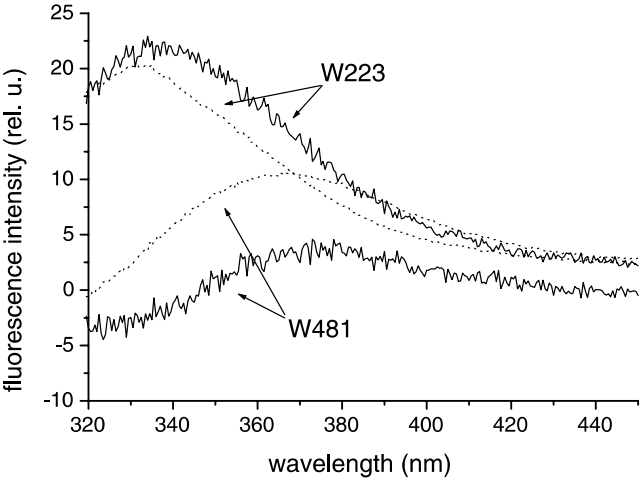

Fig. 2. Emission spectra of tryptophan residues upon excitation at 300 nm in the presence (solid line) and absence (dotted line) of yeast pMDH. Emission spectra of Trp^{223} were measured directly on the mutant containing the unique Trp residue in the position 223. Apparent spectra of Trp^{481} were obtained as a difference between spectra of the mutant containing two Trp residues in positions 223 and 481 and the spectra of the mutant containing only Trp^{223}.

W481Y) in the absence of substrate was characterized by the maximum at $\lambda = 332$ nm indicating a rather hydrophobic microenvironment of Trp^{223}. This residue becomes more exposed to the solvent after precursor binding, as deduced from the red shift of the maximum to approximately $\lambda = 337$ nm in the presence of the yeast malate dehydrogenase precursor (pMDH) (Fig. 2).

Interesting results were obtained for the mutant α-MPP-W147N containing both Trp^{223} and Trp^{481}. Subtraction of the fluorescence contribution of Trp^{223} (fluorescence of the mutant α-MPP-W147N/W481Y) from the emission spectra of the mutant α-MPP-W147N yielded apparent spectra of Trp^{481}. In the absence of any substrate the maximum at $\lambda = 357$ nm (Fig. 2) was observed, which characterizes a Trp residue fully exposed to the solvent. In addition, a negative peak in the short-wavelength part of the spectrum occurred in the presence of yeast pMDH. Because this part of the spectrum is dominated by the Trp^{223} emission, it was deduced that Trp^{223} is quenched as a donor in an energy transfer from Trp^{223} to Trp^{481} when precursor substrate is present. The precise estimation of the energy transfer efficiency was disabled due to misfolding of the mutant α-MPP-W147N/W481Y. However, the intensity decrease in the short-wavelength part of the α-MPP-W147N spectrum suggested the energy transfer efficiency of at least 16%. That means, those two Trp residues are not more than 18 Å apart in the presence of pMDH.

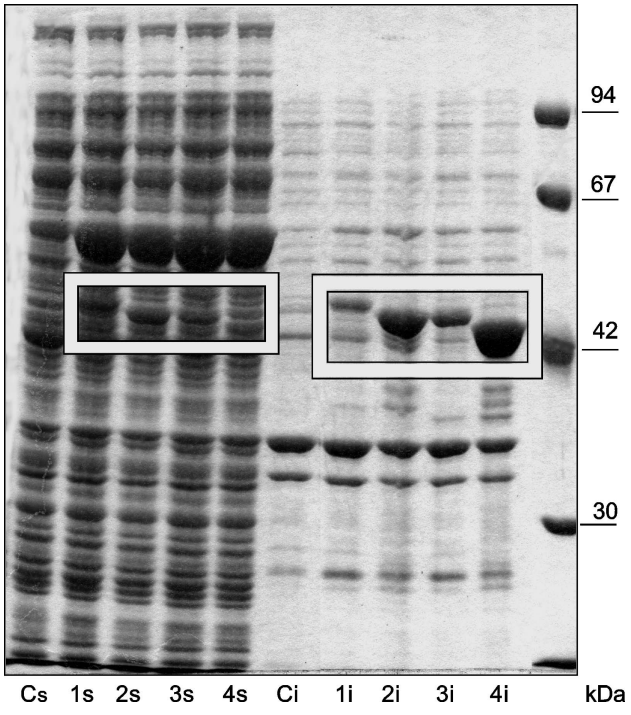

Fig. 3. Expression of the C-terminally shortened mutants of yeast α-MPP, SDS–PAGE. Mutant forms were co-expressed with chaperonins GroEL/GroES at low post-induction cultivation temperature of 12 °C. (1) His-tagged α-MPPΔC17, (2) α-MPPΔC17, (3) His-tagged α-MPPΔC30, and (4) α-MPPΔC30. C, negative control (cells transformed with pET28b vector only). s, soluble fraction, i, insoluble fraction.

Analysis of C-terminally deleted forms of α-MPP

Deletions of the C-terminal two amino acid residues (α-MPPΔC2) did not affect proper folding of the recombinant protein, i.e., the amount of protein in the soluble fraction. However, the yield of soluble α-MPPΔC17 was slightly diminished and most of the α-MPPΔC30 was present in the insoluble fraction (Fig. 3; Table 1). Moreover, the soluble portion of the His-tagged α-MPPΔC30 had no affinity to the nickel resin, suggesting improper protein folding. Consequently, it was impossible to purify either the His-tagged or native α-MPPΔC30.

The deletion of 2 and even 17 amino acid residues did not change subunit–subunit interactions (Table 1). To test interactions with β-MPP, His-tagged α-MPP mutant forms were combined with non-His-tagged β-MPP and loaded onto nickel-chelating resin.

As Fig. 4A shows, the MPP dimer with the α-subunit lacking 2 or 17 C-terminal amino acid residues showed enzymatic activity comparable to that of the wild type. However, the deletion of 30 amino acid residues results in a dramatic reduction of the activity to less than 5% of the wild type MPP in case of His-tagged α-MPPΔC30 and no activity in the native form (Fig. 4B and Table 1).

Table 1
Characteristics of C-terminally deleted forms of the yeast MPP α-subunit

Form of α-MPP	Solubility	Affinity to Ni^{2+} resin	Interaction with β-MPP	Activity	Stability
Wild type	++++	N/A	N/A	++++	++++
Wild type His-tagged	++++	+	+	++++	++++
α-MPPΔC2	++++	N/A	N/A	++++	+++
α-MPPΔC2 His-tagged	++++	+	+	++++	++++
α-MPPΔC17	++	N/A	N/A	+++	+
α-MPPΔC17 His-tagged	+++	+	+	++++	+++
α-MPPΔC30	+	N/A	N/A	–	N/A
α-MPPΔC30 His-tagged	++	–	?	+/–	N/A

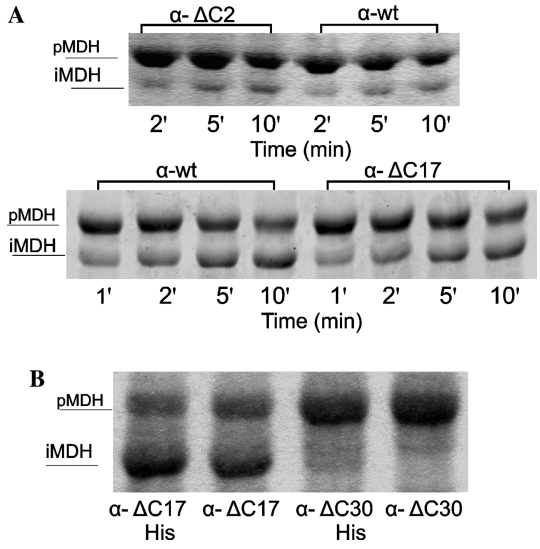

Fig. 4. Processing of pMDH by MPPs containing C-terminally shortened forms of α-MPP, SDS–PAGE: (A) Comparison of His-tagged wild type α-MPP with His-tagged α-MPPΔC17 and His-tagged α-MPPΔC2. Precursor was incubated with 20 ng MPP as described in the legend of Fig. 1. (B) Comparison of α-MPPΔC17 with α-MPPΔC30 including their His-tagged forms. Cell-free extracts containing α-MPP truncated forms were equimolarly mixed with β-subunit to yield 100 ng MPP dimers. Precursor (pMDH) was incubated with these dimers at 30 °C for 30 min. p, precursor form, i, intermediate form.

Finally, any shortening of the C-terminus caused a considerable increase in protein accessibility to degradation by trypsin. After 10 min of trypsin treatment, about 80% of wild type remained in the mixture, whereas about 60% of α-MPPΔC2 and less than 5% of α-MPPΔC17 were present (Fig. 5). Interestingly, a His-Tag at the N-terminus of the shortened mutants stabilized their structures (Table 1).

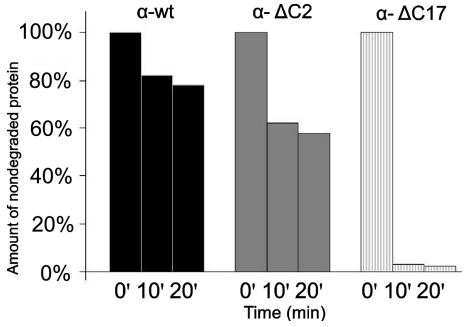

Fig. 5. Comparison of trypsin digestion accessibility of wild type α-MPP with α-MPPΔC17 and α-MPPΔC2. Portion of non-degraded protein (%) is related to starting amount (100%). Wild type or mutant forms of the α-MPP were incubated for 0, 10, and 20 min. Digestion was performed in 80 μl reaction mixture containing 10 μg α-MPP or mutant form and incubated with trypsin at 30 °C as described in Materials and methods. Aliquots containing 1 μg α-MPP were withdrawn at 0, 10, and 20 min and analyzed by SDS–PAGE.

Discussion

Although Trp^{223} as well as the whole variable interdomain linker is not conserved in the primary sequence alignment of all known α-MPPs, its substitutions led to misfolded mutant recombinant proteins. However, the adjacent α-helix terminating N-domain, immediately prior to Trp^{223}, is much conserved and could act as a hinge allowing precise conformational changes of the flexible interdomain linker.

Tryptophan fluorescence measurement of yeast α-MPP with reduced tryptophan residues, one (W147N) or two (W147N/W481Y), provided a detailed view of the α-subunit conformational change evoked by substrate, reported previously [13]. Translocation of both, the N- and C-terminal, domains of α-MPP as well as the conformational change of the linker connecting both domains were demonstrated. Trp^{223}, buried into the hydrophobic core of the subunit (Fig. 6), becomes more exposed to the solvent in the presence of substrate. It could reflect relaxation of the flexible interdomain linker accompanying the radical overall subunit conformational change during the substrate recognition process. The decrease in intensity in the short-wavelength part of the spectrum of the W147N mutant α-MPP indicates that the C-terminal penultimate Trp^{481} residue approaches to the Trp^{223} localized at the border of the N-terminal domain and interdomain linker. The distance Trp^{223}–Trp^{481} does not exceed 18 Å for the yeast substrate. Estimation of the Trp^{223}–Trp^{481} distance without

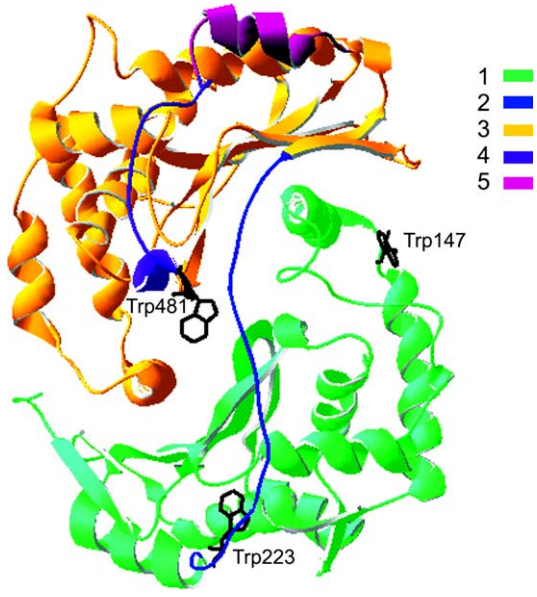

Fig. 6. Structure of the yeast MPP subunit α. Protein Data Bank with ID code 1HR6 [8]. Figure was generated by Swiss-PdbViewer software. Colors: (1) N-domain, (2) interdomain linker, (3) C-domain, (4) C-terminal 17 amino acid residues, and (5) C-terminal 30 amino acid residues. Putative positions of the C-terminal 12 amino acid residues missing in the crystal structure were completed by modeling.

any precursor substrate is impossible, but it should be longer, at least 23 Å (corresponding to 5% energy transfer efficiency, which could be considered to be our detection limit). Moreover, the Trp^{223}–Trp^{481} distance cannot be estimated from the crystal structure of yeast MPP [7], as positions of the C-terminal 12 amino acid residues 471–482 are missing there. Nevertheless, even if the missing flanking region is modeled along the inter-domain linker towards Trp^{223} (Fig. 6), the approach of both tryptophan residues could be hardly closer than ~25 Å, which is in a good agreement with the measurement. The closer approach at the presence of the substrate corresponds to a short transition state of α-MPP distinct from both states demonstrated in the crystal structures [7], i.e., without any substrate or with substrate fixed at the active site immediately before processing.

Both monitored Trp residues are localized out of the substrate binding cavity of the enzyme, thus, not directly involved in any crucial region primarily interacting with the substrate. But, their mutual movement reflects and fairly describes the conformational change evoked by the substrate binding. The MPP region responsible for the primary substrate–enzyme contact and the precise mechanism of substrate entrance into the enzyme cavity has not yet been clarified. Our data indicate that the crucial region responsible for the primary contact with substrates should be a part of the α-subunit. Also, the α-subunit alone seems crucial for probable subsequent step(s) of the enzyme action, accompanied by a radical change of the overall α-MPP conformation.

Consequently, we propose a putative mechanism of MPP action: (1) A specific region of α-MPP is responsible for the weak primary interaction with the presequences of protein substrates, which corresponds to previous findings obtained by various approaches [11–13]. Some of the highly conserved regions of α-MPP (e.g., glycine-rich region localized at the entrance into the MPP dimer cavity) are candidates of the "primary contact site." (2) The weak primary contact of the substrate with the specific region of α-MPP acts as the release pulse of the short but distinct overall conformational change of α-MPP. The documented α-MPP conformational change could relate to the initiation of the presequence entrance to the polar cavity of the dimer. The initial two steps of the enzyme–substrate interaction or at least their considerable moments are open to observation for α-MPP alone. (3) In cooperation of both MPP subunits, the presequence is fully pulled into the cavity, directed towards the active site and bound with high affinity [8] in an extended conformation at the active site. This moment is well documented in the crystal structures of the synthetic substrate peptides co-crystallized with mutant MPP deficient in the cleavage function [7]. (4) The precursor is processed and finally, the cleaved-off presequence is released from the enzyme cavity.

The data obtained from the tryptophan fluorescence measurements indicate considerable complicity of the extreme C-terminus of yeast α-MPP in the overall subunit conformational change. The C-termini of all known α-MPP sequences are highly variable without any common sequence homology. Yet, all α-MPP sequences coming from non-plant sources (yeast, *Neurospora*, *Blastocladiella*, rat or human) are slightly extended if compared to sequences of their appropriate homologous β-MPP counterparts. Moreover, demonstrating the truncation of the yeast α-MPP C-terminal 41 amino acid residues led to a loss of binding and processing activity [10] emphasizing the importance of that variable region for MPP function. In that case, one of six parallel β-sheets forming the core of the α-subunit C-domain was deleted together with the extreme C-terminus and led to destruction of the overall protein architecture. However, even a shorter deletion (ΔC30), not affecting any β-sheet structures but deleting the last short α-helix Val^{456}–Ala^{462}, had the same effect documenting the essential importance of this short supporting structural element. It was impossible to test the α-MPPΔC30 affinity to the β-MPP counterpart, regarding α-subunit improper protein folding resulting among others in a non-exposed His-tag. Probably the loss of the subunit–subunit binding capacity [10] is a consequence of the improper protein folding. The other two shorter deletions (ΔC17 and ΔC2), affecting only the region without any definable secondary structure, conserved the activity but destabilized the protein slightly (Δ2 AA) or dramatically (Δ17 or Δ30 AA). This suggests that the extreme C-terminus of α-MPP provides mechanical support to the C-terminal domain of the protein during its extensive conformational change accompanying the substrate recognition process.

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