Reconstitution of Mitochondrial Processing Peptidase from the Core Proteins (Subunits I and II) of Bovine Heart Mitochondrial Cytochrome \( bc_1 \) Complex

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Mature core I and core II proteins of the bovine heart mitochondrial cytochrome \( bc_1 \) complex were individually overexpressed in \( Escherichia coli \) as soluble proteins using the expression vector pET-I and pET-II, respectively. Purified recombinant core I and core II alone show no mitochondrial processing peptidase (MPP) activity. When these two proteins are mixed together, MPP activity is observed. Maximum activity is obtained when the molar ratio of these two core proteins reaches 1. This indicates that only the two core subunits of the \( bc_1 \) complex are needed for MPP activity. The properties of reconstituted MPP are similar to those of Triton X-100-activated MPP in the bovine \( bc_1 \) complex. When Rieske iron-sulfur protein precursor is used as substrate for reconstituted MPP, the processing activity stops when the amount of product formation (subunit IX) equals the amount of reconstituted MPP used in the system. Addition of Triton X-100 to the product-inhibited reaction mixture restores MPP activity, indicating that Triton X-100 dissociates bound subunit IX from the active site of reconstituted MPP. The aromatic group, rather than the hydroxyl group, at Tyr\(^{57} \) of core I is essential for reconstitutive activity.

Most nuclear-encoded mitochondrial proteins are synthesized on cytoplasmic ribosomes as larger precursors with presequences for targeting into mitochondria (1). These presequences are proteolytically removed during or after import of the precursors into mitochondria. Three types of processing peptidases are involved in removal of the presequence from precursors: mitochondrial processing peptidase (MPP)\(^{1} \) (2), mitochondrial intermediate peptidase (3), and inner membrane protease I (4, 5).

MPP cleaves all or part of the presequence as the initial processing step. Many proteins are mature after a one-step cleavage by MPP. Mitochondrial intermediate peptidase catalyzes a second-step cleavage in the two-step processing of some precursor proteins. The inner membrane protease I cleaves intermediate forms of proteins routed to the intermembrane space. The last two peptidases act sequentially after cleavage of the matrix targeting sequences by MPP. Thus, MPP plays an important role in the proteolytic processing of precursor proteins in the mitochondria.

MPP is located in the matrix of fungal and mammalian mitochondria and in the inner membrane of plant mitochondria (2). Matrix-localized MPP has been studied extensively and purified to homogeneity from \( Neurospora crassa \) (6), \( Saccharomyces cerevisiae \) (7), and rat liver (8, 9). Purified, matrix-localized MPP contains two nonidentical subunits, \( \alpha \)-MPP and \( \beta \)-MPP, each with molecular mass of around 50 kDa. The cDNAs encoding \( \alpha \)- and \( \beta \)-MPP from these three sources have been cloned, sequenced (6, 7, 9–13), and overexpressed in \( Escherichia coli \) cells (13–15). MPP is classified in the pitrilysin family (16) of zinc metalloproteases because of the presence of an inverted zinc binding motif, HXXEH\( _x \)H, in the \( \beta \)-MPP (17). Processing activity requires both subunits because recombinant \( \alpha \)-MPP and \( \beta \)-MPP alone show no activity and activity is restored upon mixing the two subunits (13–15). Although the role of each subunit is largely unknown, accumulating evidence suggests that \( \beta \)-MPP is the catalytic subunit (18, 19) and that both \( \alpha \) and \( \beta \)-MPP are involved in substrate binding (20, 21).

Although a wealth of information has been generated from the study of matrix-localized MPP, less is known about membrane-localized MPP. MPP activity associated with the inner mitochondrial membrane was first observed by Braun et al. (22) in potato tuber and by Eriksson and Glaser (23) in spinach leaf in 1992. Purification of plant MPP revealed that the enzyme constitutes an integral part of the \( bc_1 \) complex of the respiratory chain (22–24). Because of the sequence homology (17, 24) and immunological similarity between the subunits of matrix-localized MPP and the core subunits of the plant \( bc_1 \) complex (17, 25, 26), plant MPP activity is thought to be associated with the core proteins of the \( bc_1 \) complex. The core I subunit corresponds to \( \beta \)-MPP and core II subunit to \( \alpha \)-MPP. Traditionally the term core protein has been used to describe the two high molecular mass protein subunits of the \( bc_1 \) complex. In contrast to matrix-localized MPP, recombinant plant core I and core II produced in \( E. coli \) are reconstitutively inactive (24, 27). This failure limits the study of membrane-associated MPP at the \( bc_1 \) complex level.

Recently, MPP activity was detected in the bovine heart mitochondrial \( bc_1 \) complex after Triton X-100 treatment (28). Based on the three-dimensional structure of this complex (29, 30), the lack of MPP activity in the crystalline bovine complex was thought to be due to the binding of an inhibitor polypeptide (subunit IX) to the active site of MPP, which is located at the interface of core I and core II (28). Triton X-100, at concentrations that disrupt the structural integrity of the \( bc_1 \) complex as
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indicated by the loss of its electron transfer activity, weakens the binding of inhibitor polypeptide (subunit IX) to the active site of MPP in core subunits and thus restores MPP activity (28).

Subunit IX of the bovine bc₁ complex is the 73-amino acid presequence of ISP (31, 32). This presequence is removed in one-step and remains as a subunit (subunit IX) in the bc₁ complex (32). This is different from the process in S. cerevisiae (33) and N. crassa (34), where the ISP presequence is removed in two steps and then degraded in vivo by another, as yet unidentified, protease.

The Triton X-100-activated MPP in the bovine complex (MPP/bc₁) has properties similar to MPP detected in the plant bc₁ complex (28). They are completely inhibited by metal ion chelators such as EDTA and the EDTA-inhibited activity can be partially restored by addition of divalent cations. The cleavage site specificity of activated MPP/bc₁ depends more on the length of the amino acid sequence in the mature protein portion than on that in the presequence portion, when a synthetic peptide composed of N-terminal residues of mature protein and C-terminal residues of its presequence is used as a substrate (28). This finding is inconsistent with the present popular speculation that substrate recognition by MPP requires only structural elements in the presequence (35–41).

Our structure-function study of the MPP/bc₁ complex requires reconstitutively active core I and core II subunits of the bovine complex. There are two ways to obtain purified core subunits: by biochemical resolution of the bc₁ complex or by gene expression to generate recombinant core I and core II. The availability of the cDNA sequences of bovine core I and core II (Ref. 42 and GenBank™ accession number X59692) together with our past experience in overexpressing soluble active mitochondrial electron transfer proteins in E. coli (44–46) encouraged us to generate core I and core II by gene expression. The pET expression system was used because it introduces a His6 tag upstream from the N terminus of the expressed protein. This allows a one-step purification of recombinant protein with Ni-NTA gel. Herein we report the construction of the expression vectors, pET-1, pET-2, and pET-3pISP for core I, core II, and Rieske iron-sulfur protein precursor (pISP), respectively; growth conditions for overexpression of the active soluble form of core I, core II, and pISP in E. coli; reconstitution of MPP/bc₁ with purified recombinant core I and core II; and properties of reconstituted MPP. The inhibitory effect of subunit IX on reconstituted MPP using pISP as substrate is examined. The structural importance of Tyr57 of core I in reconstitution is investigated.

EXPERIMENTAL PROCEDURES

Materials—TA Cloning kit was from Invitrogen. Tog DNA polymerase, T4 DNA ligase, and restriction endonucleases were obtained from either MegaPro or Life Technologies, Inc. Ampicillin, tetracycline, cytomegalovirus, isopropyl-β-D-thiogalactopyranoside (IPTG), Triton X-100, betaine, and sorbitol were from Sigma. Centricon-30 and centrifrap-30 were from Amicon. LB agar, LB broth base, yeast extract, and selected peptone were from Life Technologies, Inc. Nitrocellulose membranes were from Schleicher & Schuell. Oligonucleotides were synthesized by the DNA/Protein Core Facility at Oklahoma State University. Antibodies against bovine bc₁ complex and ISP were generated in rabbits and Rieske iron-sulfur protein precursor (pISP), respectively; reconstitution of MPP/bc₁ with purified recombinant core I and core II; and properties of reconstituted MPP. The inhibitory effect of subunit IX on reconstituted MPP using pISP as substrate is examined. The structural importance of Tyr57 of core I in reconstitution is investigated.

DNA Manipulation and DNA Sequencing—General molecular genetic techniques were performed according to procedures described in Sambrook et al. (47). DNA sequencing was performed with an Applied Biosystems model 373 automated DNA sequencer at the Recombinant DNA/Protein Resources Facility at Oklahoma State University.

Polypeptide Substrates—Polypeptides composed of varying lengths of C-terminal regions of presequences and varying lengths of N-terminal regions of precursors were expressed by subcloning subunits VIII and IX sequences into the Recombinant DNA/Protein Resource Core Facility at Oklahoma State University. These synthetic polypeptides are: VPASVRYSDTDK (48, 49); ASVRSHTDKYPFDSYRRRVPFDL (50, 51); and RILPVSASVSNLVPVSRSHTDVKPDP (52, 53). Construction of E. coli Strains Expressing Core I, Core II, and the pISP and HindIII-1341-base pair DNA fragment encoding mature core I and the 1320-base pair BamHI-HindIII fragment encoding mature core II were amplified from a bovine heart cDNA library by polymerase chain reaction (PCR) using two synthetic primers: 5′-CAATTGATACCCGCCACCGGCTACCGC-3′ (the sense primer) and 5′-AAGTCGTAGACGGCAGCCA-3′ (the antisense primer) for core I; and 5′-GATCTGTCCTCTCAAGTTGCT-3′ (the sense primer) and 5′-AAGCTTCAACTCATCAGGTAAG-3′ (the antisense primer) for core II. The 530-base pair BamHI-HindIII cDNA fragment encoding pISP was amplified from the pGEM/ISP plasmid (31) by PCR using two synthetic primers: 5′-TCTGGATATCCATGGTTCGTCGTTGC (the sense primer) and 5′-GCGGAACTTACCAACCTACCATCAGTAC (the antisense primer).

PCR amplification was performed in a minicycler from M. J. Reckoning. The thermal cycle was set as follows: step 1, 95 °C for 1 min for initial denaturation; step 2, 94 °C for 1 min for denaturation; step 3, 50 °C for 2 min for annealing; and step 4, 70 °C for 2 min for extension. A total of 30 cycles were performed with a final extension step of 5 min. PCR products were confirmed by agarose gel electrophoresis and cloned into pCR2.1 cloning vectors from Invitrogen to generate pCI, pCII, and pCIII, respectively. The inserts in these plasmids were confirmed by DNA sequencing.

The NcoI-HindIII fragment from pCRI, the BamHI-HindIII fragment from pCRII, and the BamHI-HindIII fragment from pCRII were ligated into their respective sites in PET30a(+)-vector to generate pET-I, pET-II, and pET-III, respectively, which were transformed into BL21(DE3) cells by electroporation. E. coli transformants producing core I, core II, or pISP were identified by immunological screening of colonies with antibodies against core I, core II, or ISP, respectively.

Isolation of Recombinant His₆-tagged Core I, Core II, and pISP—25 ml of overnight culture of E. coli BL21(DE3)/pET-I, E. coli BL21(DE3)/pET-II, or E. coli BL21(DE3)/pET-III was used to inoculate 500 ml of TB medium containing 100 mg/ml ampicillin, 25 mg/ml kanamycin, and incubated at 37 °C with vigorous shaking until OD₆₀₀ nm reached 0.7. The culture was cooled to about 27 °C, and IPTG was harvested at a final concentration of 0.5 mM to induce synthesis of recombinant proteins. Cells were grown at 27 °C for 3 h before being harvested by centrifugation at 8,000 × g for 30 min. About 1.8 g of cell paste/500-ml culture was obtained and stored at −20 °C until use.

pISP and cell paste were washed in 20 ml of 0.2 M sodium phosphate buffer, pH 8.0, containing 300 mM NaCl (NaCl-P, buffer, pH 8.0). Cells were broken with a Fisher Ultraspec cell dismuter and treated with aliquots of phenylmethylsulfonl fluoride (100 mM in absolute alcohol) to a final concentration of 1 mM. The suspension was gently stirred for 30 min on ice and centrifuged at 30,000 × g for 25 min. About 50% of the expressed core I, core II, or pISP in E. coli was recovered in the supernatant, which was mixed with 4 ml of Ni-NTA-agarose slurry equilibrated with NaCl-P, buffer, pH 8.0. The slurry mixture was slowly shaken for 1 h at 4 °C and packed into a column (1.5 × 20 cm) that was washed, in sequence, with the NaCl-P, buffer, pH 8.0, until the A₂₈₀ nm in the column effluent dropped to less than 0.01, NaCl-P, buffer, pH 6.0, containing 10% glycerol (10 column volumes), and NaCl-P, buffer, pH 8.0, containing 50 mM imidazol (10 column volumes). Recombinant core protein was eluted from the column with 20 ml of NaCl-P, buffer, pH 8.0, containing 250 mM imidazol and dialyzed against 50 mM Tris-HCl, pH 8.0, containing 0.25 mM sucrose, overnight, to remove NaCl and imidazol. The dialyzed sample was concentrated by centrifron-10 and frozen at −80 °C until use.

Preparation of the His₆ Tag Containing Fragment from Recombinant pISP, Core I, and Core II—The His₆ tagged Recombinant pISP was mixed with 0.5 unit of recombinant enterokinase in 50 ml of cleavage buffer (20 mM Tris-HCl, pH 7.4, containing 50 mM NaCl and 2 mM CaCl₂). After the mixture was incubated at 20 °C for 30 min, 50 ml of enterokinase capture agarose slurry equilibrated with cleavage buffer was added. The mixture was incubated for 5 min and centrifuged to remove enterokinase captured agarose gel. The cleavage products, un-
tagged pISP and the His$_6$-tagged fragment containing 44 amino acid residues, and uncleaved His$_6$-tagged pISP was recovered in the supernatant fraction. When necessary, untagged pISP was separated from the His$_6$ tag containing fragment and uncleaved His$_6$-tagged pISP by Ni-NTA gel. Removal of the His$_6$ tag-containing fragment from recombinant core I and core II was accomplished in the same way as described for obtaining untagged pISP.

**Construction of E. coli Strains Expressing Core I Mutants**—Core I DNA mutations were generated by site-directed mutagenesis using the Altered Sites™ Mutagenesis system from Promega. A 1362-base pair KpnI-HindIII fragment was excised from pET1 plasmid and cloned into the KpnI and HindIII sites of pSELECT-I vector to generate pSELECT/I. The single-stranded pSELECT/I was used as the template in the mutagenesis reactions. The mutagenic oligonucleotides used were as follows: Y57T, AAGGGGCTGGACATTTTGGGAGCATCG; Y57H, GGGGCTGGCCACTTTGTGGAG; Y57F, GGGGCTGGCTTCTTGTGGAG; and Y57W, GGGGCTGGCTTTTTGTGGAG. Each of these oligonucleotides was used in combination with an ampicillin repair oligonucleotide and annealed to the single-stranded pSELECT/I.

A 1362-base pair KpnI and HindIII fragment containing mutated core I was excised from pSELECT/I and cloned into KpnI and HindIII sites of pET vector to generate pET/lano, which was then transformed into BL21 cells. Mutations were confirmed by DNA sequencing of both pSELECT/lano and pET/lano. Transformants expressing the core I$_{ano}$ protein were identified by immunological screening of colonies with antibodies against the bovine cytochrome $bc_1$ complex.

**Enzyme Preparations and General Biochemical Techniques**—Bovine heart mitochondrial cytochrome $bc_1$ complex was prepared and assayed as previously reported (48). MPP/bc$_1$ activity was measured with two substrates: synthetic peptides and pISP. When peptide substrate was used, the substrate disappearance and product formation was determined by HPLC (28). When recombinant pISP was used, the production formation and substrate disappearance was measured by SDS-PAGE. Protein concentration was routinely determined by the Bradford assay (29), using a kit from Bio-Rad; for more accurate determination of protein concentration and substrate disappearance was measured by SDS-PAGE.

**Effect of Induction Growth Conditions on Production of Recombinant Core I and pISP**—To facilitate the study of MPP/bc$_1$, recombinant core I, core II, and pISP were generated. Core I and core II are for reconstitution studies and pISP is for studies of MPP/bc$_1$ inhibition by subunit IX, because pISP is the natural substrate for MPP/bc$_1$ and the cleaved product is subunit IX. Production of core I, core II, and pISP by E. coli was first attempted with the pGEX system because this system was successfully used to overexpress functionally active QPc-9.5 kDa of the beef $bc_1$ complex (44), QPs1 (45) and QPm3 (46) of beef succinate-Q reductase, and subunit IV of the Rhodobacter sphaeroides $bc_1$ complex (53) in E. coli. Unfortunately, the expression levels of core I, core II, and pISP were low in this system. Taking the advantage of the commercially available His$_6$-tagged polypeptide expression system, we used the pET system to express beef heart mitochondrial core I, core II, and pISP in E. coli and isolated the expressed proteins by a one-step purification with Ni-NTA gel.

Production of recombinant His-tagged core I, core II, or pISP depends on IPTG concentration, induction growth time, medium, and temperature. The yield increases as the IPTG concentration and induction growth time are increased, reaching a maximum at 0.5 mM IPTG and 3 h post-induction growth. When cells are grown for more than 3 h, the total yield decreases and degradative products increase, as determined by Western blotting using antibodies against their respective proteins.

Although the expression level for these three proteins in E. coli is high using LB medium at 37 °C (accounts for 30% of the total cellular protein), about 95% of the expressed protein is in inclusion body precipitate. Solubilization of inclusion bodies of recombinant His$_6$-tagged proteins with 8 M urea followed by dialysis and Ni-NTA column chromatography yielded only small amounts of inactive soluble proteins. Because it has been reported that including betaine and sorbitol in the induction growth medium and lowering the induction growth temperature greatly increases the yield of soluble expressed protein in E. coli (44, 46), these induction conditions were adopted. About 40% of the expressed core I, core II, and pISP is in the soluble form when IPTG induction growth is in LB medium, containing 0.44 M sorbitol and 2.5 M betaine at 27 °C for 3 h. About 6 mg of purified recombinant His$_6$-tagged core I, 5 mg of core II, and 6 mg of pISP were obtained from 500 ml of their respective cell cultures.

The purified, recombinant His$_6$-tagged core I, core II, and pISP show single protein bands with apparent molecular masses of 55, 51, and 40 kDa, respectively (Fig. 1). These are larger than the protein masses of core I and core II in mitochondrial $bc_1$ complex and of pISP calculated from its protein sequence because of the addition of 44 amino acid residues to the N termini of mature core I and core II and of pISP during genetic manipulation. These extra residues are, in sequence, a His$_6$ tag, thrombin recognition sequence, S tag, and enterokinase recognition sequence. Removal of these extra residues from recombinant His$_6$-tagged core I, core II, and pISP to generate their respective untagged proteins can be achieved by enterokinase digestion followed by Ni-NTA column chromatography. The enterokinase used is removed from the digestion mixture with an enterokinase-capture gel. The uncleaved, His$_6$-tagged core I, core II, or pISP and cleaved residues are separated from the untagged proteins on a Ni-NTA column. However, the yields of untagged, mature core I and core II and pISP obtained by this method are low, mainly because of the low efficiency of enterokinase digestion (about 30%) and non-specific binding of untagged proteins to the Ni-NTA column.

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between core I and core II. Because His6-tagged recombinant reconstitution process may convert some inactive recombinant are reconstitutively inactive. The freeze-thaw step included in from their inclusion body precipitates, the resulting proteins when recombinant beef heart core I and core II are purified. recombinant core I and core II.

The His6-tagged recombinant core I and II—Purified recombinant core I and core II alone have no MPP activity. When they are mixed together MPP activity is reconstituted. The efficiency of reconstitution increases by 20% if the mixture is subjected to a freezing (∼80 °C for more than 30 min) and thawing process. The reconstituted MPP activity increases as the molar ratio of core I/core II increases (Fig. 2). Maximum activity is obtained when the molar ratio reaches 1, the same as that in bovine bc1 complex activated by Triton X-100, when calculation are based on the core I and core II content of the complex. This result indicates that reconstituent core I and core II are active, MPP activity detected in Triton X-100-treated complex is associated with core I and core II, and the structural integrity of the bc1 complex is not required for MPP/bc1 activity.

The reported failure of reconstitution of MPP from potato core proteins produced in E. coli (24) may have resulted from improper unfolding-refolding of the expressed protein produced as inclusion body precipitate rather than a need for the structural integrity of the complex for MPP/bc1 activity. In fact, when recombinant beef heart core I and core II are purified from their inclusion body precipitates, the resulting proteins are reconstitutively inactive. The freeze-thaw step included in reconstitution process may convert some inactive recombinant core I or core II to an active form or may enhance interaction between core I and core II. Because His6-tagged recombinant core I and core II have the same reconstitutive activity as untagged proteins, a structural requirement for amino acid residues near the N terminus of these two protein is not critical. Addition of 44 amino acid residues at the N terminus of both proteins has no ill effect on reconstitution. This is in line with the structural arrangement of the N termini of these two core proteins in the bc1 complex revealed from the x-ray crystallography (29).

Properties of Reconstituted MPP—Fig. 3A compares the time course of product peptide generation by reconstituted MPP from recombinant core I and core II and Triton X-100-activated MPP in the bovine complex. When a substrate peptide made of 5 residues from the C-terminal end of the precursor and 20 residues from the N-terminal end of mature ISP (−V20) is incubated with reconstituted MPP at 37 °C, product peptide formation increases with reaction time, but at a decreasing rate and reaching a maximum after 12 h. Under identical conditions, product peptide formation by Triton X-100-activated MPP in the bc1 complex reaches a maximum after 24 h. The specific activity of reconstituted MPP, calculated based on product peptide generation in the first 2 h of reaction time, is approximately twice that of Triton X-100-activated MPP in core proteins of the bc1 complex.

Fig. 3B shows the substrate peptide concentration-dependent processing activity of reconstituted MPP. When reconstituted MPP activity is measured with increasing concentrations of substrate peptide −V20, the activity increases with substrate peptide concentration. If one takes the concentration of substrate peptide that gives half of maximum activity as the apparent $K_m$, then the $K_m$ for −V20 for reconstituted MPP is 33.7 μM. Under identical assay conditions, the apparent $K_m$ for
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Although both MPP/bc₁ and yeast (or other matrix) MPP are probably zinc metallopeptidases, the role of Zn²⁺ in these two classes of MPPs should be different. The activity of MPP/bc₁ is stimulated by (25%) but not totally dependent on the addition of Zn²⁺, whereas yeast MPP has an absolute requirement for Zn²⁺.

The substrate specificity of reconstituted MPP was tested with three synthetic peptides composed of various lengths of C-terminal presequence and N-terminal sequence of mature subunit V (the iron-sulfur protein): V₁₅₋₂₀, V₁₀₋₂₀, and V₁₋₁₀. Only when V₁₋₂₀ is used as substrate is a product peptide obtained that has an N terminus corresponding to that of mature subunit V. This result agrees with those obtained with Triton X-100-activated MPP/bovine bc₁, which showed that cleavage site specificity of MPP depends more on the length of the amino acid sequence from the mature protein portion and less on the presequence portion (28).

The optimal assay conditions for reconstituted MPP are 15 mM Tris-Cl buffer, pH 8.0, at 37 °C, similar to those described for Triton X-100-activated MPP/bovine bc₁. In 15 mM Tris-Cl buffer, the activity of reconstituted MPP is not affected by addition of KCl up to 100 mM but decreases at higher salt concentrations.

The Effect of Subunit IX on Processing Activity of Reconstituted MPP—Based on the structure of the bovine heart mitochondrial bc₁ complex, it was suggested (28) that binding of subunit IX to the active site of MPP, located in the interface of core I and core II, explains the lack of MPP activity in this complex. If this suggestion is correct one should observe end product inhibition of reconstituted MPP when ISP precursor protein is used as substrate, because subunit IX is the presequence of ISP. Alternatively, the processing activity of recon-

**FIG. 4. Assay of reconstituted MPP using His₆-tagged and untagged pISP as substrate.** A. lane 1, molecular mass standards (phosphorylase B, 107 kDa; bovine serum albumin, 74 kDa; ovalbumin, 49.3 kDa; carbonic anhydrase, 36.4 kDa; soybean trypsin inhibitor, 20.9 kDa; and lysozyme, 20.9 kDa); lane 2, 5 µg of His₆-tagged pISP; lane 3, 5 µg of His₆-tagged pISP incubated with 4 µg of reconstituted MPP; lane 4, 25 µl of enterokinase-free pISP; lanes 5–7, enterokinase-free pISP was incubated with 1, 2, and 4 µg of reconstituted MPP; lane 8, 2 µg of mitochondrial bc₁ complex. B. Western blot of the samples in A. Antibodies against bovine ISP were used as first antibody and protein A-horseradish peroxidase conjugate as second antibody.
Fig. 5. Time course of product generation by reconstituted MPP using pISP as substrate in the presence and absence of Triton X-100. Lane 1, molecular mass standards (phosphorylase B, 107 kDa; bovine serum albumin, 74 kDa; ovalbumin, 49.3 kDa; carbonic anhydrase, 36.4 kDa; soybean trypsin inhibitor, 29.5 kDa; and lysozyme, 20.9 kDa); lane 2, 25 µl of enterokinase-free pISP preparation; lanes 3–5, 25-µl of enterokinase-free pISP incubated with reconstituted MPP in the presence of 0.1% Triton X-100 at 30 °C for 30, 60, and 120 min, respectively; lanes 6–8, 25 µl of enterokinase-free pISP incubated with reconstituted MPP in the absence of 0.1% Triton X-100 at 30 °C for 30, 60, and 120 min, respectively; lanes 9 and 10, samples from lane 8 were added (Triton X-100 to 0.1%) and incubated for 1 and 2 h, respectively. All samples were subjected to SDS-PAGE.

Table II

Effect of the amino acid replacement at Tyr^57^ of core I on the reconstituted activity of MPP

| Core I mutants | Reconstituted MPP activity % |
|---------------|-----------------------------|
| Wild type     | 100                         |
| Y57H          | 100                         |
| Y57T          | 0                           |
| Y57F          | 98                          |
| Y57W          | 99                          |

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...stituted MPP should be inhibited by the addition of purified or recombinant subunit IX when substrate peptide is used. The successful overexpression of pISP in E. coli, using the pET system, enables us to use recombinant pISP as substrate to examine the processing kinetics of reconstituted MPP.

When His-tagged pISP (Fig. 4, lanes 2 and 2') is used as substrate for reconstituted MPP, no cleavage is observed (Fig. 4, lanes 3 and 3'). However, when enterokinase-free, untagged pISP (Fig. 4, lanes 4 and 4'), obtained by enterokinase digestion of His-tagged pISP followed by treatment with enterokinase-capture gel, is used as substrate, processing activity is observed (Fig. 4, lanes 5 and 5'). These results indicate that the N-terminal sequence of pISP is important for processing by reconstituted MPP. This is in line with the report that the structural elements at the N-terminal region of the presequence are important for processing by MPP (37–41).

When a constant amount of untagged pISP is used as substrate for varying amounts of reconstituted MPP, the amount of product formed, subunits V and IX, increases as the amount of reconstituted MPP in the system is increased (Fig. 4, lanes 5–7 and 5'–7'). However, the reaction stops when the amount of product formed equals the amount of reconstituted MPP used, suggesting that the reconstituted MPP catalyzes only one turnover.

To further confirm that reconstituted MPP shows end product inhibition, the processing reaction was carried out in the presence and absence of 0.1% Triton X-100 (Fig. 5). In the presence of Triton X-100, the amount of product formed increases as the reaction time increases until the substrate is exhausted (Fig. 5, lanes 3–5). In the absence of Triton X-100, the reaction stops when the amount of product formed equals the amount of reconstituted MPP used (Fig. 5, lanes 6–8). These results are those predicted for end product inhibition and for Triton X-100 prevention subunit IX binding to the active site of MPP (28).

In the absence of Triton X-100 one of the cleaved products, subunit IX, remains bound to the active site of MPP, rendering the enzyme inactive. Because reconstituted MPP exhibits only one turnover, the amount of product formation should equal the amount of reconstituted MPP used in the system. In the presence of Triton X-100, subunit IX cannot bind to the active site, no inhibition is observed, and the reaction proceeds until pISP is exhausted. Triton X-100 not only prevents binding of subunit IX to the active site of reconstituted MPP but also dissociates bound subunit IX from the active site. Addition of Triton X-100 to a subunit IX inhibited system (as in lane 8 of Fig. 5) restores the processing until the substrate disappears (Fig. 5, lanes 9 and 10).

To further confirm that the observed resumption of processing activity is due to dissociation of bound subunit IX from the active site of reconstituted MPP by Triton X-100, the enzyme kinetics of inhibited reconstituted MPP treated with Triton X-100 was examined with Y57V,20 as substrate. The amount of product peptide produced by inhibited reconstituted MPP treated with Triton X-100 increases with reaction time until substrate peptide is exhausted. As expected, no product peptide generation is observed with inhibited MPP without Triton X-100 treatment. These results confirm that Triton X-100 dissociates bound subunit IX from the active site of reconstituted MPP.

The Effect of Mutation at Tyr^57^ of Core I on Reconstituted MPP Activity—Unlike MPPs from other sources, which contain an inverted zinc-binding motif, HXXEHX_{79}E, in the β-subunit, beef heart mitochondrial MPP has the Y^{57}XXEHX_{79}E sequence motif in the core I subunit. Mutational analysis indicate that both histidines and the distal glutamate in the HXXEHX_{79}E motif of β-subunit are zinc binding residues and constitute the active site of MPP (18, 19). Because in the beef core I, the first histidine in this motif is a tyrosine, it is of interest to see whether replacing this tyrosine (Tyr^57^) with histidine will increase enzyme activity. As shown in Table II, MPP activity reconstituted from Y57H mutant core I and wild type core II is the same as that reconstituted from wild type core I and core II. The yield and purity of all the recombinant mutated core I protein are comparable with those of wild type core I protein. This suggests that tyrosine and histidine play similar roles in the active site of MPP. Replacing Tyr^57^ with phenylalanine or tryptophan does not affect the reconstitutive activity of core I, whereas replacing Tyr^57^ with threonine completely abolishes its reconstitutive activity. This suggests that the aromatic ring,
rather than hydroxy group, at the position 57 of core I is essential for its reconstitutive activity.

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