Characterization and Submitochondrial Localization of the α Subunit of the Mitochondrial Processing Peptidase from the Aquatic Fungus Blastocladiella emersonii

CÍNTIA RENATA COSTA ROCHA† AND SUELY LOPES GOMES*

Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, São Paulo 05599-970, Brazil

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In an effort to investigate the molecular mechanisms responsible for the drastic morphological changes the mitochondria go through during the life cycle of the aquatic fungus Blastocladiella emersonii, the gene encoding the α subunit of the mitochondrial processing peptidase (α-MPP) was isolated. Nucleotide sequence analysis revealed that the predicted α-MPP polypeptide comprises 474 amino acids with a calculated molecular mass of 51,900 Da, presenting a characteristic mitochondrial signal sequence. Northern blot analysis indicated a single 1.4-kb transcript encoding the B. emersonii α-MPP, whose levels decrease during sporulation, becoming very low in the zoospore, and increase again during germination. Despite these variations in mRNA concentration, B. emersonii α-MPP protein levels do not change significantly during the life cycle of the fungus, as observed in Western blots. Experiments to investigate the submitochondrial localization of B. emersonii α-MPP and β-MPP were also carried out, and the results indicated that both subunits are associated with the mitochondrial inner membrane, possibly as part of the bc1 complex, as described for plants.
MATERIALS AND METHODS

Cloning of the α-MPP gene. Two degenerate oligonucleotide primers were designed on the basis of the conserved 3'-TCAGCTTCCTKWSYCTTATCAT-3' (based on two highly conserved amino acid sequences of the α-MPP from N. crassa, S. cerevisiae, rat, and potato (7, 18, 21, 23), according to the B. emersonii codon preference, and used to amplify genomic DNA by PCR (32). DNA was amplified with Taq DNA polymerase on a Gene Amp PCR system 2400 (Perkin-Elmer) with the following settings: 35 cycles of 1 min at 95°C, 2 min at 50°C, and 2 min at 72°C, followed by one 6-min extension step at 72°C. A 296-bp fragment was amplified and cloned into pUCBM21 (Boehringer Mannheim) and M13mp19 by using the EcoRI and HindIII restriction sites present in the oligonucleotides (shown in boldface type).

To isolate the complete α-MPP gene, a partial genomic library was constructed in the vector pUCBM21 (Boehringer Mannheim), with B. emersonii DNA fragments obtained from the region of the agarose gel that hybridized with the probe obtained by PCR. The library contained DNA fragments of 4 to 6 kb, obtained from a digestion with SacI, and was analyzed by colony hybridization under high-stringency conditions with a probe consisting of the PCR fragment labeled with 32P by random-primer synthesis (34). The nitrocellulose filters were prehybridized for 2 h at 37°C in 60 mM potassium phosphate (pH 6.2) containing 3× SSC (45 mM sodium citrate, 450 mM NaCl), 10 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), 50% formamide, and 5% nonfat dried milk. Hybridization was performed overnight at 37°C in the same solution after addition of the denatured probe (106 cpm/ml). The filters were sequentially washed at 37°C in 2× SSC–0.1% SDS, 1× SSC–0.1% SDS, 0.5× SSC–0.1% SDS, and 0.1× SSC–0.1% SDS for 1 h each. The filters were air dried and exposed to Kodak X-Omat film with an enhancing screen at −80°C.

DNA sequence analysis. The 4.7-kb SacI fragment containing the α-MPP gene was subjected to endonuclease restriction analysis, and several restriction fragments were subcloned into M13mp18 and M13mp19 (Bethesda Research Laboratories) for DNA sequence analysis in both strands. The nucleotide sequence was obtained by the dideoxynucleotide chain termination method with the Sequenase DNA-sequencing kit (Amersham). Analysis of sequence data and sequence comparisons were performed with programs devised by Lipman (36).

Primer extension mapping of the transcription start site. An 18-nucleotide (nt) primer, complementary to nt +146 to +163 of the α-MPP coding region, was 5'-end-labeled with [γ-32P]ATP and T4 polynucleotide kinase and hybridized with 50 μg of total B. emersonii RNA isolated from vegetative cells, zoospores, or cells that had germinated for 90 min. The annealing reaction was carried out in 25 μl of 100 mM pipervicine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0)–1 mM NaCl–5 mM EDTA at 52°C for 16 h. The nucleic acids were ethanol precipitated and resuspended in 49 μl of 50 mM Tris-HCl buffer (pH 8.3)–5 mM MgCl2–40 mM KC1–2 mM dithiothreitol–0.2 mM (each) dATP, dCTP, dGTP, and dTTP–40 U of RNase inhibitor (Boehringer Mannheim). The annealed primers were extended at 37°C for 90 min with 25 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim). RNA was digested for 30 min at 37°C by the addition of 1 μg of RNase A (Sigma), and the extended products were analyzed by polyacrylamide gel electrophoresis (PAGE) (7 M urea–7.5% polyacrylamide) by autoradiography of the gels. The transcripts were size resolved to a dideoxy sequencing ladder of the M13mp18 clone containing the 5' region of the α-MPP gene, using the same 18-nt oligonucleotide as the primer.

Preparation of antigen and immunization. The 296-bp PCR-amplified fragment was ligated into the vector pUCBM21 (Boehringer Mannheim) containing the restriction sites EcoRI (5') and HindIII (3'), which was cloned into the vector EcoRI-HindIII-digested pET21a-vector (Novagen) under the control of the T7 RNA polymerase. Overnight cultures of BL21 DE3 cells transformed with the pET21a-α-MPP plasmid were diluted 1:200 into 2× TYP medium (29) supplemented with 0.4% glycerol and ampicillin (100 μg/ml) and grown at 37°C to an optical density of 0.600 nm at 0.4. The expression of the fusion protein was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.14 mM, and growth was continued for 24 h. Cells were harvested by centrifugation at 4°C for 10 min at 5,000 × g, and the cell pellets were frozen and stored at −20°C. A bacterial lysate was prepared by thawing and resuspending the cells in 10 mM Tris-HCl (pH 7.0) containing 100 mM NaCl, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 50 μg/ml aprotinin (Sigma). The suspension was sonicated on ice with a Branson sonicator. After centrifugation for 10 min at 5,000 rpm (in a GSA rotor [Sorvall]), the pellet containing most of the α-MPP fusion protein was resuspended in buffer A (50 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM PMSF) containing 2% deoxycholate and the suspension was incubated for 10 min at room temperature. After centrifugation at 12,000 × g for 15 min, the pellet obtained was washed twice with buffer A containing 2% deoxycholate. The resulting pellet was then resuspended in buffer A containing 0.3% sodium N-lauroyl sarcosine (SDS) and left for 40 min at room temperature. The suspension was then centrifuged at 12,000 × g for 10 min at 4°C. The fusion protein was soluble in the supernatant containing 0.3% SLS, and analysis by SDS-PAGE (25) showed that it was 90% pure. A female rabbit was then immunized with approximately 200 μg of purified α-MPP fusion protein in buffer A containing 0.3% SLS and 0.5 ml of Freund's complete adjuvant. After 4 weeks, the rabbit received a second injection containing 200 μg of the antigen in Freund's incomplete adjuvant. Eight days after the second injection, the rabbit was bled from the ear, and the serum obtained was tested in Western blots.

Western blot analysis. Synchronized cells from different stages of Blastocladia life cycle were isolated as previously described (30). Cell extracts were obtained by the procedure outlined by Silva et al. (44), and proteins were resolved by SDS-PAGE (25) and then transferred to nitrocellulose membranes, as described by Towbin et al. (46). The protein quantification was done both by the Bradford method (4) and by staining the nitrocellulose membrane with Ponceau S, to make sure that equal amounts of protein were loaded in each lane of the gel. The membranes were analyzed as described previously (2), except for the use of the ECL enhanced-chemiluminescence kit (Amersham).

Carbonate extraction. Sodium carbonate treatment of membrane fractions was performed to solubilize peripherally-bound proteins. A total of 2 × 106 zoospores were resuspended in 600 μl of cold lysis buffer (100 mM Tris-HCl [pH 7.0] containing 50 mM NaCl, 1 mM β-mercaptoethanol, 1 mM PMSF, and 50 μM antipain), and the suspension was centrifuged for 10 min at 1,000 × g to eliminate unbroken cells. The supernatant was centrifuged at 100,000 × g for 10 min, and the resulting pellet was resuspended in 100 mM Na2CO3, (pH 11.5). The sample was vortexed for 1 min and centrifuged at 100,000 × g for 10 min. All the steps were carried out at 4°C. The final pellet was resuspended in 50 μl of lysis buffer, and the subfractions were analyzed by SDS-PAGE and immunoblotting, as described above.

Northern blot analysis. Total RNA from synchronized B. emersonii cells, isolated at different stages of the fungal life cycle as described above, was prepared by the method of Maniatis et al. (29) and fractionated by electrophoresis on a 1.5% agarose–2.2 M formaldehyde gel. The resolved RNAs were then blotted to Hybond N+ membranes (Amersham). Before transfer to the membrane, the gel was washed with ethidium bromide to check for the integrity of the RNA. The hybridization probe used was the 296-bp PCR-amplified fragment. As a control, the Northern blot was also hybridized to a 32P-labeled cDNA clone encoding rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (15), which was previously shown to be constitutively expressed in B. emersonii (30). The purified fragments were radioactively labeled by random-primed synthesis (14). When the PCR fragment was used as a probe, the filter was hybridized under high-stringency conditions (0.12 M Na2HPO4–NaH2PO4 buffer [pH 7.2]–0.25 M NaCl–7% SDS–1 mM EDTA at 37°C for 16 h). The filter was sequentially washed with 1× SSC–0.1% SDS at 42°C, 0.5× SSC–0.1% SDS at 42°C, and 0.1× SSC–0.1% SDS at 50°C for 30 min each. When rat GAPDH cDNA was used as a probe, the washing procedure was changed to low-stringency conditions, consisting of three sequential washes in 3× SSC–0.1% SDS at 42°C for 30 min each.

Nucleotide sequence accession number. The nucleotide sequence of the B. emersonii α-MPP gene has been submitted to the GenBank/EMBL data bank under accession no. U30568.

RESULTS

Isolation and sequence analysis of the B. emersonii α-MPP gene. By using two oligonucleotide primers designed based on highly conserved amino acid segments of the α-MPPs previously described (7, 18, 21, 23) and PCR, a 296-bp B. emersonii genomic fragment was amplified. The PCR product (Fig. 1) was cloned and sequenced. The complete 296-bp fragment encoding a portion of the B. emersonii α-MPP, was then 32P labeled by random-primed synthesis and used as a probe to screen a B. emersonii partial genomic library constructed in the pUCBM21 vector, using Blastocladia DNA fragments isolated from the region of the gel that hybridized in Southern blots to the PCR probe, as described in Materials and Methods. A positive clone presenting a 4.7-kb SacI fragment was
isolated (Fig. 1) and subjected to nucleotide sequence analysis. A total of 2,122 bp, encompassing an open reading frame of 1,535 bp, as well as 378 and 209 bp of 5' and 3' noncoding regions, respectively, was sequenced. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 2.

The predicted \( B. \) emersonii \( \alpha \)-MPP contains 474 amino acids with a calculated molecular mass of 51.9 kDa, a value comparing very well with the molecular mass of 52 kDa determined by SDS-PAGE. The initiator methionine was chosen based on the presence of an in-frame stop codon (TAA) immediately preceding it.

FIG. 2. Nucleotide sequence of the \( B. \) emersonii \( \alpha \)-MPP gene and the deduced amino acid sequence. Capital letters indicate deoxynucleotides in exons or sequences upstream and downstream of the coding region of the gene; lowercase letters show the deoxynucleotides in the intron. Nucleotide +1 denotes the A of the ATG of the initiator methionine. Residues preceding it are indicated by negative numbers. The deduced amino acid sequence is shown below the nucleotide sequence. The arrows (↓) indicate putative signal sequence cleavage sites. A probable polyadenylation signal is shown in bold. The predicted \( B. \) emersonii \( \alpha \)-MPP contains 474 amino acids with a calculated molecular mass of 51.9 kDa, a value comparing very well with the molecular mass of 52 kDa determined by SDS-PAGE. The initiator methionine was chosen based on the presence of an in-frame stop codon (TAA) immediately pre-
ceding the ATG. A putative mitochondrial signal sequence, which is rich in basic and hydrophobic amino acids, has been identified (40). Two possible cleavage sites have been observed, one between residues Ser-14 and Thr-15 and another between Leu-20 and Pro-21 (Fig. 2). In both cases an arginine residue (Arg-13 and Arg-19) is present at position 2 relative to the processing site, which is a common theme of such sites (37, 50, 51). The first putative cleavage site seems to be the most probable, since it retains the amino acid Leu-20 in the mature protein, which is a residue conserved in all \( \alpha \)-MPPs previously characterized (Fig. 3).

B. emersonii \( \alpha \)-MPP second-structure prediction, using the NNPREDICT program (24, 31), showed a strong tendency for the formation of an \( \alpha \)-helix between Ala-5 and Thr-15, followed by an indefinite secondary structure until Glu-73. The presence of an \( \alpha \)-helical structure in the amino-terminal region of \( \alpha \)-MPP is in agreement with the prediction made for the putative signal peptide from B. emersonii \( \beta \)-MPP (39) and also fulfills the requirements for the MPP to recognize its substrates (40).

A single intron, 113 bp in size, is found within the \( \alpha \)-MPP coding region, interrupting the highly conserved sequence encoding the amino acid sequence LAFKSTH. The 5' and 3' splice sites follow the consensus for B. emersonii introns (9), with a probable branch site (CTGAC) located between nucleotides 1271 and 1275.

The Blastocladiella \( \alpha \)-MPP presents an overall sequence identity of 40.7% to the \( \alpha \)-MPP from N. crassa (42), 36.1% to the \( \alpha \)-MPP from S. cerevisiae (21, 38), 34.7% to P-55 from rat...
(34), and 32.6% to subunit III of cytochrome c reductase from Solanum tuberosum (7). It displays the conserved amino acid essential for catalytic activity, the His-58 of the putative metal-binding motif HFLEK (45), and the four cysteine residues (Cys-89, Cys-170, Cys-319, and Cys-338) conserved in all the α-MPPs from fungi (Fig. 3). These cysteine residues could play a functional role in the fungal protein, since they are not conserved in plants and mammals (42, 45). The Blastocladiella putative metal-binding motif presents a serine (Ser-61) in a position where a negatively charged aspartic acid or glutamic acid is usually found (Fig. 3). Nevertheless, Striebel et al. (45) have shown by mutation analysis that replacing the E-112 of rat α-MPP by a glutamine residue, which is its uncharged derivative, improved the performance of MPP beyond wild-type level.

Characterization of the transcription initiation site and analysis of the 5′ noncoding region. Primer extension experiments were performed to determine the start site of transcription of the α-MPP gene, and the results are shown in Fig. 4A. A single start site was observed at position −126 from the ATG encoding the initiator methionine. The predicted transcription start point is indicated by an arrow. The putative core sequences representing the binding sites for the TATA-binding protein (TATA box), Sp1 (GC box), CTF/NF1 (CCAAT box), and helix-loop-helix transcription factor (E box) are indicated.
mentary to nt +146 to +163 of the coding region (Fig. 4B), was 5’ end labeled with [γ-32P]ATP and hybridized with total RNA isolated from Blastocladiella vegetative cells, zoospores, or germling cells. The hybrids were then extended with avian myeloblastosis virus reverse transcriptase, and the extension products were resolved by PAGE with urea. The fragments were sized by comparison to a dideoxy sequencing ladder of M13mp19 containing the 3.3-kb SacI-XhoI genomic fragment (Fig. 1), with the same oligonucleotide as primer. Detailed examination of the 5’ noncoding region of the Blastocladiella α-MPP gene (Fig. 4B) revealed the presence of some elements usually found in eukaryotic promoters. A putative TATA box was located about 50 nt upstream of the single transcription start site (nt −175 to −181). There was an excellent consensus sequence for a CCAAT box (nt −273 to −281). One copy of the hexanucleotide CCGCCC was also observed (nt −32 to −37). This hexanucleotide is identical to the core sequence recognized by mammalian transcription factor Sp1 (11). Two copies of the consensus core sequence for helix-loop-helix (CANNTG) transcription factor-binding sites (33) were found at positions −90 to −95 and −142 to −147.

Expression of the α-MPP gene at the mRNA and protein levels. Northern blot analysis was performed to investigate possible changes in the levels of the mRNA encoding the α-MPP during the B. emersonii life cycle. Total RNA isolated from synchronized cells at 0, 60, 90, 120, and 180 min of sporulation, zoospores, or cells at 45 and 90 min of germination was resolved by agarose gel electrophoresis, transferred to Hybond N+ membrane, and probed with the 32P-labeled PCR fragment. A single 1.4-kb transcript encoding the α-MPP, whose levels decreased during sporulation, reaching almost undetectable levels in the zoospore stage, and increased again during germination was observed (Fig. 5). As a control, the same filter was hybridized to a heterologous cDNA probe encoding rat GAPDH, which is constitutively expressed during the life cycle of this fungus (30).

To investigate the levels of the α-MPP protein throughout the B. emersonii life cycle, a specific polyclonal antiserum was obtained from a rabbit immunized with a fusion protein, corresponding to about 15 kDa of the central portion of B. emersonii α-MPP, fused to a histidine tag at the NH2 terminus. Total extracts from synchronized cells, isolated at different times during the B. emersonii developmental cycle, were subjected to Western blot analysis with the α-MPP-specific antiserum. A single 52-kDa band, whose levels did not change significantly during the life cycle of the fungus, was recognized by the antiserum (data not shown). Thus, even though α-MPP mRNA levels vary during B. emersonii life cycle, the amount of the corresponding protein does not change appreciably.

Subcellular localization of α-MPP and β-MPP in B. emersonii. To determine the subcellular localization of B. emersonii α-MPP and β-MPP, total zoospore extracts were fractionated by ultracentrifugation (100,000 × g for 10 min). The soluble fraction was saved, and the particulate fraction was subjected to alkaline treatment with Na2CO3. This procedure, which has been used to investigate the topological organization of the components of S. cerevisiae cytochrome bc1 complex (3), discriminates between peripheral and integral membrane proteins, solubilizing the former ones. In S. cerevisiae, this treatment led to the complete solubilization of the core I and core II proteins, whereas subunits VII and VIII were not extracted from the mitochondrial membrane.

The different fractions obtained from zoospore extracts were analyzed by Western blotting with antiserum against B. emersonii α-MPP and β-MPP, anti-Hsp60 antiserum (Sigma), antiserum against subunit VII of the cytochrome bc1 complex of S. cerevisiae, and antiserum against ATPase of B. emersonii. The results, shown in Fig. 6, indicate that α-MPP is completely associated with the insoluble fraction of the extract but can be solubilized by the sodium carbonate treatment. β-MPP was associated partially with the insoluble fraction (60%) and partially with the soluble fraction (40%); it could be totally solubilized by the sodium carbonate treatment. The Hsp60, which in all organisms studied is localized in the mitochondrial matrix, has been detected only in the soluble fraction. This last result indicates that the giant mitochondria of the zoospores were disrupted during cell extract preparation. The B. emersonii P-type ATPase, which is an integral membrane protein, was shown to be totally associated with the particulate fraction and was not solubilized after the alkaline treatment with Na2CO3.

The antiserum against the subunit VII of cytochrome bc1 complex cross-reacted with a B. emersonii 14-kDa polypeptide, in agreement with the expected molecular mass for this conserved subunit (20). The putative B. emersonii subunit VII was
observed to be completely associated with the insoluble fraction of the zoospore extract and was only partially solubilized after the alkaline treatment (Fig. 6). This result is consistent with the localization of this subunit inside the bc₁ complex, whose three-dimensional structure has been determined both by electron microscopy of membrane crystals and X-ray crystallography of the purified complex from N. crassa (22) and bovine heart (53), respectively.

**DISCUSSION**

The *B. emersonii* α-MPP gene encodes a precursor protein encompassing 474 amino acids, with a calculated molecular mass of 51,000 Da. The ATG assigned as encoding the initiator methionine is preceded by an in-frame stop codon (TAA). A putative mitochondrial signal sequence has been identified, rich in basic and hydroxylated amino acids and with the potential to form an amphiphilic α-helix. The coding region is interrupted by a single intron, 113 bp in size, located in a conserved α-MPP domain (encoding LAFKSTH) present in all organisms. The conservation of this intron in other fungi, such as *N. crassa* or *S. cerevisiae*, is present only in the mitochondrial matrix. Nevertheless, α-MPP and β-MPP protein levels do not change significantly during the *B. emersonii* life cycle, which indicates that the drastic morphological changes in the mitochondria in this fungus do not lead to functional alterations, at least in relation to the processing of mitochondrial presequences.

The analysis of α-MPP and β-MPP secondary structure, using the TMBase program (19), has indicated that α-MPP presents two domains (amino acids 279 to 294 and amino acids 325 to 347) with a high score for the formation of a transmembrane helix, whereas β-MPP does not present sequences with such characteristics. These structural predictions agree well with the data obtained in the subcellular localization experiments. The fact that *B. emersonii* α-MPP, like the putative subunit VII of the bc₁ complex, is found only in the particulate fraction and that the former is completely solubilized after sodium carbonate treatment whereas the latter is also partially solubilized suggests that α-MPP is associated with the mitochondrial inner membrane like subunit VII but less strongly. On the other hand, 60% of *B. emersonii* β-MPP has been found in the particulate fraction and was solubilized by the sodium carbonate treatment. These results, in combination, indicate that α-MPP is peripherally associated with the mitochondrial inner membrane whereas the β-MPP is probably anchored to the α-subunit.

The localization of *B. emersonii* MMP in the inner mitochondrial membrane differs from what was observed in other fungi such as *N. crassa* or *S. cerevisiae*. In *S. cerevisiae*, both protease subunits are found soluble in the matrix, whereas in *N. crassa*, even though β-MPP is found partially associated with the membrane fraction and partially soluble in the matrix, α-MPP is present only in the mitochondrial matrix.

*B. emersonii* MMP localization instead resembles the situation observed in plants, where both protease subunits are integrated in the bc₁ complex, replacing the core I and core II proteins (12, 13). According to Braun and Schmitz (5), who proposed that the core proteins of the bc₁ complex are evolu-
tionary relics of a processing protease, plants exemplify the original situation. Our results suggest that B. emersonii MPP could, as in plants, be a bifunctional protein, playing a structural role in the assembly of the bc1 complex besides its function as the MPP. This hypothesis is strengthened by the fact that Southern blot analysis presented no evidence of other genes similar to the α-MPP and β-MPP genes in B. emersonii (results not shown) and by the position of this fungus at the base of the fungal phylogenetic tree, right where the branching of fungi, animals, and plants occurred (49).

Irreproachable confirmation that the processing activity is integrated into the bc1 complex in B. emersonii will be possible only with the demonstration that the isolated bc1 complex catalyzes the processing of mitochondrial signal sequences; this study is presently under way.

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