Purified Inner Membrane Protease I of Yeast Mitochondria Is a Heterodimer*

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Inner membrane protease I is bound to the outer face of the yeast mitochondrial inner membrane and mediates the proteolytic maturation of cytochrome b$_2$ and cytochrome oxidase subunit II. We have previously shown that one of its subunits is a 21.4-kDa integral membrane protein encoded by the nuclear IMP1 gene. We have now purified the active protease approximately 300-fold from yeast mitochondria. It has an apparent molecular weight of 35,000 and contains not only Imp1 but also a previously unrecognized 19-kDa subunit. Partial amino acid sequencing identifies this subunit as the product of the recently described IMP2 gene (Nunnari, J., Fox, T., and Walter, P. (1993) Science 262, 1997-2004).

EXPERIMENTAL PROCEDURES

Transport of proteins across membranes is usually accompanied by proteolytic removal of targeting sequences from the transported precursor polypeptides (1). This process is catalyzed by a group of enzymes referred to as translocation pro tease. These proteases represent a heterogenous group which includes soluble as well as membrane-bound members. Recent sequence studies have revealed striking similarities between three types of membrane-bound translocation proteases: leader peptidase of the bacterial plasma membrane, signal peptidase of the endoplasmic reticulum, and inner membrane protease I of the yeast mitochondrial inner membrane (2-4). These enzymes have been collectively termed "type I signal peptidases." As the translocation protease associated with the thylakoid membrane of chloroplasts has a substrate specificity similar to that of bacterial leader peptidase (5), it may represent a fourth member of this group.

Even though type I signal peptidases share at least one similar subunit, their subunit composition differs. Whereas bacterial leader peptidases are generally monomers (2, 6, 7), signal peptidases of the endoplasmic reticulum may contain two to five subunits depending on the organism (8, 9). The subunit compositions of inner membrane protease I has remained uncertain. Since overexpression of the 21.4-kDa Imp1 subunit was not accompanied by increased enzyme activity, we had suggested that the active protease is a heterooligomer (10).

We have now partially purified inner membrane protease I from yeast mitochondria and show that the Imp1 subunit is tightly associated with a 19-kDa protein. As the apparent molecular weight of the active enzyme (35,000) is close to the sum of the molecular weights of these two polypeptides, the protease appears to be a heterodimer.

Immunopurification of Inner Membrane Protease I

Preparation of Protein A-Sepharose Antibody Matrix—Five ml of protein A-Sepharose (Pharmacia, Uppsala, Sweden) was incubated for 2 h at room temperature with 20 ml of rabbit antiserum against Imp1. The antibody matrix (20 ml) was washed successively with five times 11.3-ml volumes of 0.01 M Tris-HCl (pH 7.5), 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5 M NaCl, and 0.5 M NaCl. The supernatant was adjusted to 10%.

Images were imported from the Biozcentrum, University of Basel, Switzerland. The images were then cropped and resized as necessary. The text was then added using a natural language processing tool.
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**Table 1**

<table>
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<tr>
<th>Protein</th>
<th>Implp antigen</th>
<th>Yield antigen</th>
<th>Purification antigen</th>
<th>Activitya</th>
<th>Specific activity</th>
<th>Yield activity</th>
<th>Purification activity</th>
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<td>(100)</td>
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<tr>
<td>Mono Q eluate</td>
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<td>61</td>
<td>353</td>
<td>0.655</td>
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<td>43</td>
<td>262</td>
<td>46.2</td>
<td>1.925</td>
<td>76</td>
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*a* Units as defined in Ref. 10.

**Fig. 1.** Purification of inner membrane protease I by anion exchange chromatography. A 2% octyl-POE extract of solubilized membranes was chromatographed on Mono Q-Sepharose as described under "Experimental Procedures." Aliquots (50 μl each) of the extract (E), the flow-through (FT), the wash (W), and of every fifth column fraction were run in duplicate on two SDS-15% polyacrylamide gels. One gel was stained with Coomassie Brilliant Blue (upper panel); the other gel was subjected to immunoblotting with an antisem to Implp (lower panel). The sizes of the molecular weight standards (St.) are given on the right. In the lower panel, a mitochondrial extract from a yeast strain overproducing Implp was used as a standard.

**Fig. 2.** Purification of inner membrane protease I by glycerol gradient centrifugation. A, the pooled Implp-containing fractions from the anion exchange chromatography step were further purified by centrifugation in a linear glycerol gradient (see "Experimental Procedures") and 2.2-ml fractions were collected. One hundred-μl aliquots of the input (IP) and 200-μl aliquots of each fraction were precipitated with 10% trichloroacetic acid and 75% of each sample was analyzed on a 13% SDS-polyacrylamide gel and staining with Coomassie Brilliant Blue (upper panel). The remaining 25% of each aliquot was run on a duplicate gel and analyzed by immunoblotting with anti-Implp antibody (lower panel). Fraction 12 was lost during sample preparation. Molecular weight standards (St.) are as in Fig. 1. B, apparent molecular weight of native inner membrane protease I. The sedimentation of Implp, as determined in panel A (vertical arrow) was compared to that of different molecular weight standards run in a separate glycerol gradient (see "Experimental Procedures"). The presence of the individual size markers in each gradient fraction was analyzed on a 13% SDS-polyacrylamide gel and staining with Coomassie Brilliant Blue. BSA, bovine serum albumin. CYT C, cytochrome c.

**Amino Acid Sequencing**

The proteins of pooled eluates from five immunopurifications (100 ml) were precipitated with 10% trichloroacetic acid, washed extensively with cold acetone, resuspended in 500 μl of 3-fold concentrated SDS-sample buffer, and resolved by preparative 13% SDS-PAGE. The gel was stained for 10 min with 0.1% Coomassie Brilliant Blue in 40% methanol, 1% acetic acid and washed five times for 5 min with water. The bands corresponding to Implp and the 19-kDa band (Impl2p) were cut out, electroeluted, and subjected to Edman degradation.
Twenty ml of pooled Implp-containing fractions from the glycerol gradient step were subjected to immunopurification on immobilized anti-Implp IgGs (see "Experimental Procedures"). Aliquots (0.5%, 50 μl) of the unfraccionated pool (P) and the flow-through (FT), as well as 20% (2 ml) of the immunoceluante (EL), were precipitated with 10% trichloroacetic acid and solubilized with 100 μl of DTT-containing 3-fold concentrated SDS-sample buffer. Thirty-five and 15 μl of each fraction was run on the same SDS-15% polyacrylamide gel. The lane between the flow-through and the eluate fractions was loaded with bovine serum albumin to avoid cross-contamination of the flanking lanes. One half of the gel containing the 35-μl samples was analyzed by silver staining (left panel), and the other half containing the 15-μl samples was analyzed by immunoblotting with anti Implp-antibody (right panel). Positions of Implp (Implp) and the 19-kDa protein (Implp2) are indicated. Molecular weight standards are indicated on the left.

**Assays**

Protein was determined using the BCA protein assay (Pierce Chemical Co.) using crystalline bovine serum albumin as the standard. All fractions containing DTT were first precipitated with 10% trichloroacetic acid to remove the reducing agent.

Inner membrane protease I activity was assayed by measuring the conversion of the cytochrome b2 intermediate to mature cytochrome b2 (10). Since wild type yeast was used for the purification, each fraction was checked for absence of mature cytochrome b2 by immunoblotting. To compare the activities at the different purification steps, it was essential to optimize the phosphatidylserine concentration in the assay mixture for each individual fraction. This concentration varied from 0.5 to 1.0 mg/ml. Inner membrane protease I activity was determined for several protein concentrations to determine the linear range of the assay. Specific activity was calculated from points in the linear range and from at least two independent assays.

Immunoblots and activity assays were quantified using a model 300 A computing densitometer (Molecular Dynamics).

**RESULTS AND DISCUSSION**

Purification of inner membrane protease I to near homogeneity was accomplished by differential solubilization of yeast mitochondria, anion exchange chromatography, glycerol gradient velocity sedimentation, and immunochromatography. Fractions from each step were monitored by assaying the cleavage of the cytochrome b2 intermediate to mature cytochrome b2 and by quantifying the Implp1 subunit by immunoblotting. Since yeast mitochondria contain only very low amounts of inner membrane protease I, we chose commercially available pressed bakers’ yeast as the starting material. As a result the solubilization protocol described here is slightly different from that used previously for mitochondria from a laboratory-grown yeast strain (10).

Differential extraction of yeast mitochondria with the non-ionic detergent octyl-POE effected a 5.7-fold purification of the Implp1 antigen with a yield of more than 90% (Table I); enrichment of the protease activity could not be determined since the enzyme could not be reliably assayed in the crude mitochondria. Anion exchange chromatography (Fig. 1) led to an additional 11-fold enrichment of the Implp1 subunit. This step also appeared to remove an inhibitor of the protease, as enzyme activity was purified 26-fold with a yield of 220% relative to the solubilized membrane fraction. Velocity sedimentation in a glycerol gradient (Fig. 2A) purified both the Implp1 antigen and the activity 3–4-fold. As the density of octyl-POE is 1, this also showed that the apparent molecular weight of the active enzyme is approximately 35,000 (Fig. 2B).

The three purification steps described above enriched the Implp1 antigen 262-fold with respect to mitochondria (Table I). Assuming that the enzyme activity cofractionates with Implp1, the active enzyme was purified more than 400-fold (76 × 5.7, Table I). The purest fractions were still inhomogeneous (Fig. 2). However, most of the remaining impurities could be removed by immunochromatography, using IgGs directed against a C-terminal peptide of Implp1. The eluate from the immunocolumn consisted mainly of two polypeptides: the 21.4-kDa Implp1 subunit and a 19-kDa polypeptide (Fig. 3). The wash did not contain either of the two proteins (not shown).

Judged from the intensity of the silver stain, these two polypeptides were not recovered in equimolar amounts. The reason for this is not clear. The two bands might well react differently with the silver stain. Alternatively, some of the Implp1 subunits might have been lost during immunochromatography; as the enzyme binds to the column only through its Implp1 subunit, elution at low pH might not completely disrupt binding of Implp1 to the IgGs, yet dissociate all of the 19-kDa subunit from its partner, Implp1.

Both subunits were subjected to N-terminal sequencing. The identity of Implp1 was readily confirmed, as the sequence obtained corresponded to residues 2–16 of the predicted Impl gene product (Fig. 4A) (3). The protein is thus imported into mitochondria without proteolytic cleavage. As the N terminus of the 19-kDa protein was blocked, we repeated the sequence analysis with tryptic fragments. One of the fragments yielded an unambiguous sequence (Fig. 4B). This sequence corresponded to positions 51–62 of Impl2p, recently described by Nunnari et al. (14). Genetic and biochemical evidence has shown that Impl2p is a type I signal peptidase which is bound to Implp1 and which catalyzes the maturation of cytochrome c1.

The present study and the results of Nunnari et al. (14) suggest strongly that inner membrane protease I is a heterodimer composed of one 21.4-kDa Implp1 subunit and one 19-kDa Implp2 subunit. This fact explains why overproduction
of Imp1p alone does not increase enzyme activity (10), and why mutations in Imp2p may block maturation of cytochrome b2, as well as of cytochrome c1 (14). The heterodimeric inner membrane protease I is thus intermediate in complexity between bacterial leader peptidase and the signal peptidase of the endoplasmic reticulum. However, our results do not exclude the possibility that, in mitochondria, inner membrane protease I is complexed to other proteins such as subunits of the protein transport channel across the inner membrane.

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REFERENCES