Signal Peptides Having Standard and Nonstandard Cleavage Sites Can Be Processed by Imp1p of the Mitochondrial Inner Membrane Protease*

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Xuemin Chen, Clint Van Valkenburgh, Hong Fang, and Neil Green‡

From the Department of Microbiology and Immunology, School of Medicine, Vanderbilt University, Nashville, Tennessee 37232-2363

We have performed a site-directed mutagenesis study showing that residues comprising the type I signal peptidase signature in the two catalytic subunits of the yeast inner membrane protease, Imp1p and Imp2p, are functionally important, consistent with the idea that these subunits contain a serine/lysine catalytic dyad. Previous studies have shown that Imp1p cleaves signal peptides having asparaginase at the −1 position, which deviates from the typical signal peptide possessing a small uncharged amino acid at this position. To determine whether asparaginase is responsible for the nonoverlapping substrate specificities exhibited by the inner membrane protease subunits, we have substituted asparaginase with 19 amino acids in the Imp1p substrate i-cytochrome (cyt) b2. The resulting signal peptides containing alanine, serine, cysteine, leucine, and methionine can be cleaved efficiently by Imp1p. The remaining mutant signal peptides are cleaved inefficiently or not at all. Surprisingly, none of the amino acid changes results in the recognition of i-cyt b2 by Imp2p, whose natural substrate, i-cyt c1, has alanine at the −1 position. The data demonstrate that (i) although the −1 residue is important in substrates recognized by Imp1p, signal peptides having standard and nonstandard cleavage sites can be processed by Imp1p, and (ii) a −1 asparaginase does not govern the substrate specificity of the inner membrane protease subunits.

The type I signal peptidase family consists of enzymes located in the plasma membranes of eubacterial cells, the endoplasmic reticulum (ER)

membrane, and the inner membrane of mitochondria. These enzymes function similarly to cleave signal peptides from the amino termini of precursor proteins after the delivery of these precursors to their appropriate cellular compartments (reviewed in Ref. 1). Based on the crystal structure of leader peptidase from Escherichia coli (2), the eubacterial signal peptides exhibit five characteristic residues that make up a type I signature sequence consisting of a serine/lysine catalytic dyad and three structurally important amino acids, an arginine and two aspartic acids, that are positioned close to the catalytic site. The ER homolog of the eubacterial signal peptidases, Sec11p, has a similar signature; however, Sec11p contains histidine in place of lysine. Because this histidine is important for catalysis (3), Sec11p may utilize a serine/histidine dyad or a catalytic triad of serine/histidine/aspartic acid. The type I signal peptidase found in the inner membrane of mitochondria consists of two subunits, both of which are catalytic (4–6). Termed inner membrane protease (IMP), sequence comparisons suggest that the IMP subunits, Imp1p and Imp2p, may contain serine/lysine dyads, like their eubacterial counterparts (1).

Imp1p from the yeast Saccharomyces cerevisiae cleaves the signal peptides from the precursors to cytochrome (cyt) b2, a nuclear encoded protein, and cyt oxidase subunit II, a protein encoded within the mitochondrion. The signal peptides of these precursors possess asparaginase at the −1 position from the cleavage site (4). The presence of a −1 asparaginase in the Imp1p substrates violates the “−1, −3 rule” proposed in previous studies (7–9), which is based on the fact that signal peptides in the eubacterial and ER systems contain small uncharged amino acids at the −1 position and, to a lesser extent, the −3 position. Indeed, site-directed mutagenesis studies have confirmed the critical role of the −1 amino acid for the correct cleavage of signal peptides in the ER and in eubacterial cells (10–12). From the crystallographic analysis of leader peptidase, the −1 and −3 amino acids probably help to position the signal peptide relative to the active site through their interactions with distinct binding pockets on the enzyme’s surface (2).

In agreement with the −1, −3 rule, Imp2p cleaves a more conventional signal peptide such as that of the cyt c1 precursor, which has alanine at the −1 position (5). A reasonable hypothesis is that Imp1p cleaves a different signal peptide from that of Imp2p is that Imp1p is able to recognize a −1 asparaginase (5). An extension of this idea is that asparaginase may fit into the appropriate binding pocket of Imp1p and be excluded from the corresponding site in Imp2p. To address this hypothesis, we have asked the following question: do mutations affecting the −1 asparaginase of the cyt b2 precursor inhibit its cleavage by Imp1p and, conversely, allow for its cleavage by Imp2p? We have also asked whether the type I signature sequences of Imp1p and Imp2p are important for their enzymatic activities.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Antibodies—Media for growing yeast (13) and glycerol-containing media (5) have been described. A rat anti-HA antibody (clone 3F10; Roche Molecular Biochemicals) was used in this study. Yeast strain JNY34 MATα imp2Δ Δ ura3–52 trp1) was obtained from Jodi Nunnari (University of California, Davis, CA). Strain XCY101 (MAT α imp1::HIS3 ura3–52 leu2–3,112 his3–Δ200 trp1::SUC2 Δ901 suc2::Δ9 lys2–80) was constructed as follows. A BamHI fragment encoding the HIS3 gene (14) was inserted into the BamHI site of IMP1, which corresponds to amino acid 97. A linear DNA fragment containing the

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‡ To whom correspondence should be addressed. Tel.: 615-343-0453; Fax: 615-343-7392; E-mail: neil.green@mcmail.vanderbilt.edu.

1 The abbreviations used are: ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; IMP, inner membrane protease; cyt, cytochrome; HA, hemagglutinin.
IMPI gene disrupted with HIS3 was introduced by homologous recombination into the chromosome of strain SEY2210 (MAT a ura3-52 leu2-3,112 his3-A200 trp1-A901 suc2-29 y2d-80) (14). The disruption was confirmed by the fact that strain XCY101 failed to grow in glycerol-containing media, and this growth defect was complemented by plasmids bearing the wild-type IMPI gene.

Biochemical Procedures—Pulse-chase methods have been published previously (15). For immunoprecipitation analysis, the procedure published previously (15) was used, except for the following modifications. Anti-HA antibodies and protein G (instead of protein A)-Sepharose were used. An anti-HA antibody mixture (2

![Image](https://example.com/image.png)

**Fig. 1.** Regions of homology among members of the type I signal peptidase family. Box I, Box II, and Box III represent sequences that are homologous in leader peptidase from *E. coli*, SipS from *B. subtilis*, and the IMPI and IMP2 subunits of the yeast IMP. The positions of the catalytic serine and lysine of leader peptidase are indicated (+), and the amino acids important for maintaining the structure of leader peptidase are indicated (#). Gaps in these sequences are indicated by a dash.

RESULTS

Amino Acids Comprising the Type I Signal Peptidase Signature Are Important for IMPI and IMP2 Function—Five key amino acids have been found to serve critical roles in catalysis and structural maintenance of the type I signal peptidases in eubacteria, including leader peptidase from *E. coli* (2, 19–21) and SipS from *B. subtilis* (22). This signature sequence includes the catalytic dyad residues, serine and lysine, and three amino acids, an arginine and two aspartic acids, that are structurally important (Fig. 1). Because IMPI and IMP2 of the mitochondrial signal peptidase from the yeast *S. cerevisiae* contain this signature, we asked whether these five amino acids were important for enzyme function using a site-directed mutagenesis approach. Mutations altering these five residues were constructed in the IMPI gene and inserted into pHS426 (2 µm URa3) (see “Experimental Procedures”). The constructs were then introduced into yeast strain XCY101 (imp1::HIS3). The genotypes of strains used in this study are listed under “Experimental Procedures.” To determine whether these mutations inhibited enzyme activity, a pulse-chase assay was used to monitor for cleavage of the IMPI substrate cyt b2 (c-pyt b2) contains a bipartite signal sequence (23). The amino-terminal half is cleaved by the mitochondrial processing protease (aβ) within the mitochondrial matrix (24–27). This cleavage event liberates an intermediate form, i-cyt b2, that contains the second half of the bipartite signal, which is cleaved by IMPI. Cells of strain XCY101 bearing the imp1 mutations and bearing pXC2 (2 µm TRP1) that contained the wild-type CYB2 gene (encoding p-cyt b2) that had been tagged at the carboxyl terminus with the HA epitope (see “Experimental Procedures”) were grown to log phase in a glucose-containing medium and treated with a 10-min pulse using [35S]methionine and [35S]cysteine, followed by a 60-min chase with excess unlabeled methionine and cysteine. Proteins were precipitated from cell extracts using anti-HA antibodies and subjected to SDS-PAGE and fluorography.

After the 60-min chase, mature cyt b2HA (m-cyt b2HA) was present in cells carrying the wild-type IMPI gene, whereas the intermediate species, but no mature species, was found in XCY101 cells (imp1::HIS3) lacking IMPI (Fig. 2). The p-cyt b2HA protein containing the bipartite signal was not detected under these conditions. In cells carrying the S40A, S40T, K84R, K84H, D131Y, D138N, and D138E mutations, i-cyt b2HA was seen exclusively, indicating a strong inhibition of cleavage by these mutated forms of IMPI. In agreement with this result, yeast cells bearing these imp1 mutations were not viable on agar plates containing glycerol, a nonfermentable carbon source. IMPI is required for cellular respiration, probably because at least one of the IMPI substrates is nonfunctional when its signal peptide is still attached. Therefore, growth of yeast cells in the presence of a nonfermentable carbon source is an indicator of IMPI function (4, 5). The remaining imp1 mutations (D131N, D131E, and R85A) inhibited the cleavage of i-cyt b2HA less well (Fig. 1). Furthermore, yeast cells bearing these mutations were able to grow on agar plates containing glycerol as the sole carbon source. Taken together, all five amino acids comprising the type I signature of IMPI were important for function, although Asp131 could be changed to asparagine and glutamic acid without appreciable loss of activity, and Arg85 could be changed to alanine, resulting in only a partial enzymatic defect.
We next asked whether the type I signature amino acids in Imp2p were important for its function. A series of imp2 mutations was constructed by site-directed mutagenesis. The IMP2 gene and the imp2 mutations were introduced into pHF455 (2 \(\mu\)m URA3) (see “Experimental Procedures”). This plasmid series was then introduced into strain JNY34 (imp2Δ), which contained pXC1 (2 \(\mu\)m TRP1) that bore the CYT1 gene (encoding p-cyt \(c_1\) that had been tagged at the carboxyl terminus with the HA epitope) (see “Experimental Procedures”). Cells were grown in a medium containing glucose and then subjected to a 15-min pulse and a 30-min chase.

When the wild-type IMP2 gene was present in cells, a large amount of m-cyt \(c_1\)HA was exhibited (Fig. 3). These cells also displayed both p-cyt \(c_1\)HA, which contains a bipartite signal peptide (28), and i-cyt \(c_1\)HA, which contains the second half of the bipartite signal. The presence of these two immature species in cells containing wild-type Imp2p may be due to the fact that the CYT1 gene was overexpressed (see “Experimental Procedures” for a description of the plasmid used). A novel species of slightly smaller molecular mass than m-cyt \(c_1\)HA was also present in cells containing wild-type Imp2p (Fig. 3, see lane IMP2 wt). The identity of this novel form is not known; however, it may represent a proteolytic fragment arising from overexpression of the apoprotein. In JNY34 (imp2Δ) cells lacking Imp2p, m-cyt \(c_1\)HA was absent (Fig. 3). Likewise, little or no production of m-cyt \(c_1\)HA was detected in cells carrying the S41A, S41T, K91R, K91H, D124N, D124Y, D131N, and D131E mutations. One of these mutations, the S41A mutation, was also constructed in a previous study, and results similar to those reported here were obtained (5). The R92A and D124E mutations only partially inhibited the production of m-cyt \(c_1\)HA (Fig. 3).

None of these imp2 mutations inhibited the growth of JNY34 cells on agar plates containing glycerol, consistent with the fact that Imp2p activity is nonessential for cellular respiration (5). However, a physical interaction between Imp1p and Imp2p is important for the stability of Imp1p and thus for the growth of yeast cells in the presence of a nonfermentable carbon source (5). The fact that none of the imp2 mutations inhibited the growth of JNY34 cells in the presence of glycerol suggests that the imp2 mutations did not prevent the binding of Imp2p to Imp1p. Indeed, we have shown that Imp2p containing the above-mentioned mutations altering Ser\(^{41}\) or Lys\(^{83}\) was stable in yeast cells for at least 30 min, whereas mutations altering Arg\(^{92}\), Asp\(^{124}\), and Asp\(^{131}\) led to the degradation of Imp2p in vivo (data not shown).

In summary, the data show that, as with Imp1p, the five amino acids comprising the type I signature of Imp2p are important for its enzymatic activity. Considering that mutations altering Ser\(^{41}\) and Lys\(^{83}\) of Imp2p inhibit enzyme activity completely but do not affect Imp2p stability in vivo, Imp2p and, by extension, Imp1p probably contain a serine/lysine catalytic dyad.

**Imp1p Cleaves Signal Peptides Containing Standard and Nonstandard Cleavage Sites**—Imp2p cleaves the signal peptide of i-cyt \(c_1\) that contains the –1 residue, alanine, whereas Imp1p cleaves the signal peptide of i-cyt \(b_2\) that has a nonstandard –1 residue, asparagine. Because current models suggest that asparagine may provide a determinant for this substrate specificity, we prepared a series of constructs that introduced 19 amino acids into the –1 position of i-cyt \(b_2\)HA to identify amino acid substitutions that inhibit cleavage by Imp1p and promote cleavage by Imp2p. These changes were constructed by site-directed mutagenesis (see “Experimental Procedures”). To determine whether Imp1p was able to cleave signal peptides containing these amino acid substitutions, a series of plasmids was introduced into cells of strain JNY34 (imp2Δ). Because Imp1p is unstable in the absence of Imp2p, the cells also carried plasmid pXC3 that contained the imp2 (S41A) mutation. This mutation renders Imp2p enzymatically inactive (see Fig. 3) but does not affect Imp2p stability and thus Imp1p stability (5). Cells were grown to log phase and subjected to a 10-min pulse followed by a 60-min chase, and proteins were precipitated from cell extracts using anti-HA antibodies.

As shown in Fig. 4A, the placement of five different amino acids into the signal peptide of i-cyt \(b_2\)HA led to its efficient cleavage by Imp1p. Serine, cysteine, methionine, alanine, and leucine were tolerated almost as well as the naturally occurring asparagine residue. Only a small amount of cleavage of i-cyt \(b_2\)HA by Imp1p was detected when glutamine and threonine were present and no cleavage was detected when any other amino acid was present at the –1 position (Fig. 4A). The inhibition of cleavage by 14 different amino acid substitutions at the –1 position argues against the idea that cleavage can occur at a nearby asparagine when serine, cysteine, methionine, alanine, and leucine are present.

We next introduced this series of mutations into cells of strain XCY101 (imp1::HIS3), and then we performed a pulse-chase analysis to determine whether Imp2p could cleave these mutant forms of i-cyt \(b_2\)HA. As shown in Fig. 4B, Imp2p was unable to cleave i-cyt \(b_2\)HA containing any amino acid at the –1 position.

We reasoned that the introduction of a –1 methionine into i-cyt \(b_2\)HA may result in a new translational start site, which could be mistaken for cleavage at the –1 site by Imp1p (Fig. 4A). We therefore repeated the pulse-chase analysis, only this time, proteins were immunoprecipitated from cell extracts of strain JNY34 (imp2Δ)/pXC3 (imp2 (S41A)) after both the pulse and the chase. The p-cyt \(b_2\)HA protein was present after the pulse, and m-cyt \(b_2\)HA was present after a 30-min chase (data not shown). This precursor-product relationship established that a cleavage event had occurred, supporting the idea that Imp1p can cleave i-cyt \(b_2\)HA after the –1 methionine. Further support for this conclusion comes from the fact that a protein having the size of m-cyt \(b_2\)HA was absent from strain XCY101 (Fig. 4B).

The data presented in Fig. 4B show that, surprisingly, Imp2p could not cleave i-cyt \(b_2\)HA that had alanine at the –1 position. Because the natural substrate for Imp2p, i-cyt \(c_1\), contains a –1 alanine, we chose to examine the –3 residue of i-cyt \(b_2\)HA.
Recognition of Signal Peptides in Mitochondria

Both of the Imp1p substrates, i-cyt b$_2$ and p-cyt oxidase subunit II, contained isoleucine at the −3 position, whereas the Imp2p substrate had a −3 alanine. It thus seemed plausible that in order to promote cleavage of i-cyt b$_2$ by Imp2p, it may be necessary to change the −1 and −3 amino acids to alanine. To this end, we constructed a series of mutations in which different combinations of asparagine and alanine were placed at the −1 position, and different combinations of isoleucine and alanine were placed at the −3 position of i-cyt b$_2$HA. The mutations were introduced into plasmid pHF454 (2 μm TRP1) (see "Experimental Procedures").

Fig. 4. Cleavage of −1 mutant forms of p-cyt b$_2$. A series of TRP1-based plasmids encoding the cyt b$_2$HA precursor containing amino acid changes listed at the top of the figure was introduced into strain JNY34/pXC3 (imp2Δ/pXC3 (imp2 (S41A) URA3) (A) and strain JNY34/pXC3 (imp2 Δ/pXC3 (imp2 (S41A) URA3) (B). Strain JNY34/pXC3 (imp2 Δ/pXC3 (imp2 (S41A) URA3) containing the wild-type cyt b$_2$HA precursor is depicted in the first lane of B. Cells were subjected to a 10-min pulse and a 60-min chase, and proteins were precipitated using anti-HA antibodies (see "Experimental Procedures").

Fig. 5. Effect of changing the −1 and −3 amino acids of p-cyt b$_2$. A series of TRP1-based plasmids encoding the cyt b$_2$HA precursors containing the amino acid changes listed at the top of the figure was introduced into strain JNY34/pXC3 (imp2Δ/pXC3 (imp2 (S41A) URA3) (first three lanes) and strain JNY34/pXC3 (imp2 Δ/pXC3 (imp2 (S41A) URA3) (last three lanes). Cells of these strains and strain JNY34/pXC3 lacking the plasmid encoding the cyt b$_2$HA precursor (middle lane) were subjected to a 10-min pulse and a 60-min chase, and proteins were precipitated using anti-HA antibodies (see "Experimental Procedures").

In this study, we have asked whether amino acids comprising the type I signal peptidase signatures of the IMP subunits are functionally important. Studies in eubacterial systems have shown that the type I signature consists of a serine, lysine, arginine, and two aspartic acid residues that are important for the function of leader peptidase from E. coli and SipS from B. subtilis (21, 22). Imp1p and Imp2p from the yeast mitochondrion contain similar signatures (Fig. 1); however, functional studies have been lacking, except for an analysis of Ser$^4$, which has been shown to be essential to Imp2p (5). Here, we have demonstrated that conservative changes of the signature serine and lysine residues of Imp1p (Fig. 2) and Imp2p (Fig. 3) abolish their enzymatic activity. Moreover, Imp2p contains conservative changes of the signature serine and lysine residues is stable in vivo (data not shown), consistent with studies in eubacterial systems showing that the corresponding residues make up a catalytic serine/lysine dyad (2, 21, 22). In the eubacterial studies, the remaining amino acids, an arginine and two aspartic acid residues, are important structural amino acids, and from the data presented here, it is plausible that the corresponding amino acids in Imp1p and Imp2p serve a similar role.
role. Considering these results, Imp1p and Imp2p contain similar type I signatures, although they cleave different signal peptides inside the mitochon-}

Another goal of this study is to understand why Imp1p and Imp2p exhibit nonoverlapping substrate specificities. To this end, we have tested the hypothesis that the presence of asparagine at the −1 position directs signal peptides to Imp1p and away from Imp2p. We have substituted the −1 asparagine of i-cyt b2 with 19 amino acids and then examined the mutant signal peptides for cleavage by Imp1p (Fig. 4A) and Imp2p (Fig. 4B). The data reveal that five different amino acids can be introduced into the −1 position of i-cyt b2 without appreciable loss of cleavage efficiency by Imp1p. The amino acids tolerated are serine, cysteine, methionine, alanine, and leucine. Including the naturally occurring amino acid, asparagine, this group includes polar (asparagine, serine, and cysteine) and nonpolar (methionine, alanine, and leucine) amino acids and amino acids with varying side-chain lengths. Both of the sulfur-containing amino acids (cysteine and methionine) are permitted. Surpris-

The absence of efficient cleavage of 14 mutant signal peptides thus argues strongly against the hypothesis that asparagine is responsible for directing signal peptides to Imp1p and away from Imp2p. During the course of this study, we noted that Imp1p substrates i-cyt b2 and p-cyt oxidase subunit II contain a −3 isoleucine, whereas the Imp2p substrate, i-cyt c1, contains a −3 alanine. Because the −3 position is thought to be important for signal peptide recognition in the ER and eubacterial systems (2, 29–31), we constructed another series of mutations, changing the −1 and −3 amino acids of i-cyt b2. However, not even i-cyt b2 containing alanine at both the −1 and −3 positions could be cleaved by Imp2p (Fig. 5).

Thus, the goal of identifying factors that govern the substrate specificities exhibited by the IMP subunits has been achieved only partially in this study. We have shown that amino acids at the −1 and −3 positions are important for proper cleavage of the signal peptide of i-cyt b2. In contrast to previous models, however, a −1 asparagine is not required to direct signal peptides to Imp1p. Because the attachment of heme c to the i-cyt c1 apoprotein is essential for cleavage of this substrate by Imp2p (28), and the heme binding domain of i-cyt b2 is needed for its efficient cleavage by Imp1p (32, 33), current studies are focused on determining whether the mature portions of these proteins are important for their recognition by specific IMP subunits.

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