Dislocation by the m-AAA Protease Increases the Threshold Hydrophobicity for Retention of Transmembrane Helices in the Inner Membrane of Yeast Mitochondria

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Background: The m-AAA protease dislocates transmembrane segments from the mitochondrial inner membrane. The m-AAA protease dislocates transmembrane segments from the mitochondrial inner membrane.

Results: The presence of the m-AAA protease increases the hydrophobicity required for a transmembrane segment to remain in the membrane.

Conclusion: The hydrophobicity thresholds for transmembrane segment retention in the mitochondrial inner membrane differ with or without the m-AAA protease.

Significance: Retention of a transmembrane domain in the inner membrane depends on recognition by the m-AAA protease.

Sorting of mitochondrial inner membrane proteins is a complex process in which translocons and proteases function in a concerted way. Many inner membrane proteins insert into the membrane via the TIM23 translocon, and some are then further acted upon by the mitochondrial m-AAA protease, a molecular motor capable of dislocating proteins from the inner membrane. This raises the possibility that the threshold hydrophobicity for the retention of transmembrane segments in the inner membrane is different depending on whether they belong to membrane proteins that are m-AAA protease substrates or not. Here, using model transmembrane segments engineered into m-AAA protease-dependent proteins, we show that the threshold hydrophobicity for membrane retention measured in yeast cells in the absence of a functional m-AAA protease is markedly lower than that measured in its presence. Whether a given hydrophobic segment in a mitochondrial inner membrane protein will ultimately form a transmembrane helix may therefore depend on whether or not it will be exposed to the pulling force exerted by the m-AAA protease during biogenesis.

Nuclearily encoded mitochondrial inner membrane proteins are imported into the organelle through the TOM (transporter outer membrane) complex in the outer mitochondrial membrane and engage either the TIM23 or the related TIM22 translocon in the inner membrane. Some TIM23-dependent inner membrane proteins follow a “conservative sorting” pathway, in which they are first fully translocated into the matrix and then inserted into the inner membrane from the matrix side in a process that depends on the Oxa1 translocon (1, 2). Other proteins use a “stop transfer” mechanism in which the transmembrane segments exit laterally from the TIM23 translocon and integrate into the lipid bilayer (2). Finally, the mitochondrial m-AAA protease can dislocate substrate proteins from the inner membrane in an ATP-dependent manner.

Given these different pathways for the insertion and retention of transmembrane segments in the inner membrane, a key goal is to understand the sequence characteristics that distinguish between transmembrane segments that do and those that do not insert into the membrane during passage through the TIM23 translocon and, furthermore, to determine to what extent subsequent dislocation by the m-AAA protease affects membrane retention of different transmembrane segments.

In a recent study (3), we replaced a hydrophilic transmembrane segment in the model yeast inner membrane protein Mgm1 with a series of 19-residue model segments (referred to as H-segments) composed of n Leu and 19 – n Ala residues and determined the number of Leu residues required for 50% retention of the H segment in the inner membrane. The threshold was found to be unexpectedly high (n = 5–6), considerably higher than what we found previously for insertion of H-segments into the endoplasmic reticulum membrane in yeast and mammalian cells (4–6).

Because Mgm1 is not a substrate of the m-AAA protease (7), we looked for another model protein that would allow the study of membrane retention of transmembrane segments in the context of m-AAA protease dislocation activity. Mitochondrial
cytochrome c peroxidase (Ccp1) is a heme-binding protein localized in the intermembrane space. During import into the mitochondrion, the N-terminal, positively charged, matrix-targeting sequence in precursor Ccp1 (p-Ccp1) is first translocated across the outer and inner membranes, and the following hydrophobic segment is integrated into the inner membrane via the TIM23 complex (see Fig. 1A) (8, 9). The membrane-integrated form is then dislocated from the membrane and concomitantly cleaved at Ala29 in the middle of the hydrophobic segment by the inner membrane m-AAA protease, generating mature Ccp1 (m-Ccp1). In Δyta10 cells, which lack the critical Yta10 subunit of the functional m-AAA protease, the threshold for 50% retention in the inner membrane of Mgm1 H-segments is n = 0–1. Replacing the wild-type hydrophobic segment with Leu6/Ala19...H-segments thus converts Mgm1 into an m-AAA protease substrate, explaining the unexpectedly high threshold hydrophobicity observed in our previous study.

These and our previous results (3) suggest a model in which the threshold hydrophobicity for TIM23-mediated insertion in the inner membrane is n ≈ 1–2, with a considerably higher threshold of n ≈ 5–6 required to withstand the force exerted by the m-AAA protease when it extracts protein segments from the membrane.

EXPERIMENTAL PROCEDURES

Plasmid Construction—To facilitate subcloning of an H-segment into the first hydrophobic domain of CCP1, pYX142CCP1H5msal was prepared by overlap PCR (12) using pYX142CCP1 (8) as a template with the following primer pairs: 5’-GTTAATCAGAGAATTGTTG-3’ and 5’-ACCCGCCGATGAGGGGCTTGTCAGAATGGCTTC-3’. The resulting plasmid was digested with SmaI, purified, and ligated into pJK110 (3) by homologous recombination as described previously.

To assess the possible influence of the m-AAA protease on membrane retention of transmembrane segments, we determined the threshold hydrophobicity for retention in the inner membrane of Leu6/Ala19...H-segments engineered into Ccp1 in both the absence and presence of a functional m-AAA protease. Strikingly, we found that in the presence of the m-AAA protease, the membrane retention threshold for H-segments in Ccp1 is n = 5–6, but that in the absence of a functional m-AAA protease, the threshold is n = 1–2. The threshold hydrophobicity for membrane retention is thus significantly higher in the presence than in the absence of a functional m-AAA protease.

To determine the threshold hydrophobicity for H-segment retention for Ccp1 in the presence of the m-AAA protease and that found previously for Mgm1 (3) prompted us to further measure the retention of Mgm1 H-segments in the mitochondrial inner membrane in the absence of a functional m-AAA protease. Contrary to the situation for wild-type Mgm1 (7), we found that the balance between the long and short isoforms in Mgm1 H-segment constructs is strongly affected by the m-AAA protease: in a strain with a nonfunctional m-AAA protease, the threshold for 50% retention in the inner membrane of Mgm1 H-segments is n = 0–3. Replacing the wild-type hydrophobic segment with Leu6/Ala19...H-segments thus converts Mgm1 into an m-AAA protease substrate, explaining the unexpectedly high threshold hydrophobicity observed in our previous study.

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Mgm1 (Fig. 1A) to measure the degree of membrane retention of H-segments. The first hydrophobic segment of Mgm1 was replaced with Leu_u/A19_H-segments, and the degree of membrane retention was quantitated by measuring the relative levels of $l$- and $s$-Mgm1.

We applied the same approach to study the dislocation of H-segments from the mitochondrial inner membrane mediated by the m-AAA protease using Ccp1 as a test protein (Fig. 1, A and B). By replacing the Ccp1 hydrophobic domain with H-segments of the composition GGPG(L$_n$1/A$_{19-n}$)GPGG where $n = 0$–8 (see supplemental Table S1 for sequences), we determined the dislocation activity of the m-AAA protease on the H-segments using the degree of cleavage of Ccp1 by the rhomboid protease Pcp1 as a measure of membrane retention. m-Ccp1 originating from Pcp1 cleavage is formed only if the H-segment first partitions into the inner membrane and is then dislocated by the m-AAA protease. Ccp1 constructs were expressed from a low-copy plasmid in Δccp1 yeast strains containing either a functional or nonfunctional (Δccp1, Δyta10) m-AAA protease (8). Formation of dislocated, cleaved m-Ccp1 or non-dislocated, full-length p-Ccp1 was assayed by [35S]Met pulse-chase experiments, followed by immunoprecipitation with an antisera directed against Ccp1 and SDS-PAGE analysis.

m-AAA Protease Affects the Threshold Hydrophobicity for Membrane Retention of Ccp1 H-segments—Consistent with previously published results (8, 9), virtually all wild-type p-Ccp1 was converted to m-Ccp1 in the presence of the m-AAA protease (Yta10), whereas in the absence of Yta10, wild-type p-Ccp1 remained in the membrane-anchored precursor form (Fig. 2A).

Interestingly, in the presence of a functional m-AAA protease, Ccp1 variants with H-segments containing $n = 2$–5 leucines gave rise mostly to m-Ccp1 (Fig. 2, A and D), whereas in the absence of Yta10, the p-Ccp1 form predominated (Fig. 2, A and E). H-segments with $n = 2$–5 thus behaved similarly to the natural hydrophobic segment in Ccp1, partitioning into the inner membrane and from there being efficiently dislocated by the m-AAA protease, allowing Pcp1 cleavage and formation of m-Ccp1. In a functional complementation experiment, these Ccp1 variants were expressed in the Δccp1 strain and exposed to hydrogen peroxide (8). Ccp1 in its mature folded form (m-Ccp1) functions as a reactive oxygen species-binding scavenger in the mitochondrial intermembrane space, conferring resistance to oxidative conditions (16). Indeed, Ccp1 with an H-segment containing $n = 2$–4 leucines fully restored cell growth in the presence of hydrogen peroxide to levels similar to those restored by wild-type Ccp1 (Fig. 3).

It is notable that despite producing similar levels of m-Ccp1 compared with p-Ccp1 in the presence of the m-AAA protease,
compared with the construct with $n = 2$ (Fig. 2D), the Ccp1 construct with $n = 5$ could only partially restore growth of the Δccp1 strain under oxidative conditions. We have not analyzed this construct further; however, it is possible that the slightly lower amount of m-Ccp1 produced in the Ccp1 construct with $n = 5$ (Fig. 2A) might be insufficient to fully restore Ccp1 function.

In both the presence and absence of the m-AAA protease, the H-segment with $n = 0$ yielded almost exclusively p-Ccp1, whereas for $n = 1$, there was a mixture of p and m-Ccp1 (Fig. 2, A, D, and E). As shown by Tatsuta et al. (8), reducing the hydrophobicity of the hydrophobic domain in wild-type Ccp1 facilitates dislocation from the lipid bilayer and allows Ccp1 maturation by Pcp1 in an m-AAA protease-independent way. Additionally, the same authors found that complete deletion of the hydrophobic domain causes mistargeting of Ccp1 to the matrix, bypassing cleavage by Pcp1. It appears that the $n = 0$ construct and $\sim 50\%$ of the molecules in the $n = 1$ construct are mislocalized to the matrix and therefore not cleaved by Pcp1. The remaining $\sim 50\%$ of the molecules in the $n = 1$ construct are found as the m-Ccp1 form regardless of the presence or absence of the m-AAA protease (Fig. 2A), suggesting that the m-AAA protease-independent “facilitated dislocation” mechanism mentioned above may give rise to the observed m-Ccp1 form. Although none of these Ccp1 variants could fully rescue cell growth in the presence of hydrogen peroxide, yeast cells expressing the $n = 5$ construct grew at a comparable rate to cells expressing the $n = 1$ construct (Fig. 3).

Finally, Ccp1 carrying H-segments with $n = 6–8$ produced almost exclusively p-Ccp1 in both the presence and absence of a functional m-AAA protease (Fig. 2, A, D, and E). In light of the Ccp1 maturation mechanism (8), we infer that these very
hydrophobic segments partition efficiently into the inner membrane and that the m-AAA protease cannot dislocate them into the matrix.

We conclude that, in the context of Ccp1, the threshold hydrophobicity required for 50% retention of an H-segment in the inner membrane varies depending on the m-AAA protease activity. In the absence of a functional m-AAA protease, the threshold is $n_{\text{H1005}}^{\text{1–2}}$ (Fig. 2E). In contrast, in the presence of a fully functional m-AAA protease, the threshold is considerably higher at $n_{\text{H1005}}^{\text{5–6}}$ (Fig. 2D).

$m$-AAA Protease Affects the Threshold Hydrophobicity for Membrane Retention of Mgm1 H-segments—In our previous study (3), we used Mgm1 as a model protein to study TIM23-mediated insertion of H-segments into the mitochondrial inner membrane. Membrane-anchored $l$-Mgm1 is generated when the H-segment is retained in the inner membrane, whereas soluble $s$-Mgm1 is produced when the H-segment is translocated into the matrix and the following hydrophobic segment is cleaved by Pcp1 (Fig. 1A). In the Mgm1 system, the threshold hydrophobicity for 50% retention of the H-segment was found to be $n_{\text{H1015}}^{5}$, similar to the threshold obtained for Ccp1 in the presence of the m-AAA protease.

Although maturation of wild-type Mgm1 is independent of the m-AAA protease (10, 11), this unexpected correspondence between the Ccp1 and Mgm1 results prompted us to examine the involvement of the m-AAA protease in the processing of Mgm1 constructs carrying Leu$_n$/Ala$_{19-n}$ H-segments. The Mgm1 constructs were expressed in yeast strains with a functional or nonfunctional (Δyta10) m-AAA protease (14). Indeed, in the absence of the m-AAA protease, Mgm1 with an $n = 0$
m-AAA Protease Affects Retention of a Transmembrane Segment

**FIGURE 3.** Functional complementation of Δccp1 with Ccp1 H-segment constructs. Ccp1 activity was assessed by growth of a cell suspension on solid medium with filter paper (1-cm diameter) soaked with 5 μl of 10% (v/v) hydrogen peroxide on the center of the plate. The diameter (d) of the circular region around the filter paper with no visible growth after 2–3 days of incubation was measured. Percent growth rescue relative to Δccp1 cells expressing wild-type Ccp1 was calculated as follows: 100\% \left( \frac{(d_\text{WT}) - (d_\text{Δccp1} \cdot \text{Δyta10})}{(d_\text{WT})} \right), where (d_\text{WT}) is the average diameter measured for Δccp1Δyta10 cells expressing wild-type Ccp1 (WT(--)), (d_\text{Δccp1} \cdot \text{Δyta10}) is the average diameter measured for Δccp1 cells expressing wild-type Ccp1, and (d) is the average diameter measured for Δccp1 cells expressing the Ccp1 H-segment construct in question. Δccp1 cells expressing nonfunctional Ccp1 with a mutation (H242P) in the heme-binding domain (8) were included as a negative control. Means ± S.E. from at least three independent experiments are shown (error bars). 0L, zero Leu residues.

H-segment gave ~65% l-Mgm1, reaching 100% l-Mgm1 for n = 3 (Fig. 2, B and E). These results parallel those seen for Ccp1 in Δyta10 cells, strongly suggesting that Mgm1 variants by the m-AAA protease are respiratory-deficient (22) due to impaired translation and assembly of mitochondrial encoded respiratory chain complex subunits (14, 17). We therefore considered the possibility that the variation in the ratio of l- and s-Mgm1 in the Δyta10 strain may not be a direct consequence of m-AAA dislocation activity but rather results from a general mitochondrial malfunction. To this end, we expressed the Mgm1 constructs in the respiratory-deficient W303-1a rho" strain, which carries truncations in the mtDNA (23) and shows a defect in translation of mitochondrially encoded subunits of respiratory chain complexes, as does the m-AAA mutant (14, 17). No variation in the amount of l- and s-Mgm1 compared with the wild-type W303-1a strain was found (Fig. 4). We conclude that the differences in threshold hydrophobicity seen in the presence or absence of the m-AAA protease are due to a direct involvement of the m-AAA protease in the dislocation reaction.

**DISCUSSION**

We have analyzed the role of the m-AAA protease in membrane dislocation of model hydrophobic Leu_n/Ala_19−n segments (H-segments) in the context of three different proteins: Ccp1, Mgm1, and a CoxVa-Mgm1 fusion protein. Our results show that in the presence of a functional m-AAA protease, the threshold for membrane retention of H-segments is n = 5–6, whereas in the absence of the m-AAA protease, the threshold is n = 1–2. These data suggest that if the hydrophobicity of a transmembrane domain in an inner membrane protein is higher than n = 5–6, the m-AAA protease will not be able to extract it from the membrane. The unexpectedly high threshold for membrane insertion of Mgm1 H-segment constructs via the TIM23 translocon that we found previously (3) can now be explained as resulting from membrane dislocation of these Mgm1 variants by the m-AAA protease. Possibly, model proteins such as Mgm1 and CoxVa containing engineered H-segments cannot fold and/or oligomerize properly and therefore become substrates of the m-AAA protease.

Generally, the hydrophobicity of mitochondrial inner membrane proteins is rather low compared with bacterial inner membrane or eukaryotic plasma membrane proteins (25). Thus, our data suggest that most transmembrane segments cannot fold and/or oligomerize properly and therefore become substrates of the m-AAA protease.
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