ATP-dependent Proteolysis in Mitochondria

m-AAA PROTEASE AND PIM1 PROTEASE EXERT OVERLAPPING SUBSTRATE SPECIFICITIES AND COOPERATE WITH THE mtHsp70 SYSTEM*

(Received for publication, January 30, 1998, and in revised form, April 6, 1998)

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To analyze protein degradation in mitochondria and the role of molecular chaperone proteins in this process, bovine apocytochrome P450scc was employed as a model protein. When imported into isolated yeast mitochondria, P450scc was mislocalized to the matrix and rapidly degraded. This proteolytic breakdown was mediated by the ATP-dependent PIM1 protease, a Lon-like protease in the mitochondrial matrix, in cooperation with the mtHsp70 system. In addition, a derivative of P450scc was studied to which a heterologous transmembrane region was fused at the amino terminus. This protein became anchored to the inner membrane upon import and was degraded by the membrane-embedded, ATP-dependent m-AAA protease. Again, degradation depended on the mtHsp70 system; it was inhibited at non-permissive temperature in mitochondria carrying temperature-sensitive mutant forms of Ssc1p, Mdj1p, or Mge1p. These results demonstrate overlapping substrate specificities of PIM1 and the m-AAA protease, and they assign a central role to the mtHsp70 system during the degradation of misfolded polypeptides by both proteases.

Molecular chaperone proteins bind non-native protein structures and stabilize them against aggregation (1). By this means, they ensure proper folding of newly synthesized proteins, provide protection against heat denaturation, and mediate the vectorial translocation of polypeptides across biological membranes (2–7). Furthermore, evidence is accumulating that chaperone proteins play a pivotal role in ATP-dependent proteolytic processes (8–10). Chaperone and proteolytic activities thereby constitute a quality control system which prevents the deleterious accumulation of misfolded polypeptides in the cell. For instance, the degradation of misfolded polypeptides by Lon-like proteases in Escherichia coli or mitochondria depends on Hsp70 proteins which prevent the aggregation of substrate polypeptides (11–15). In addition to classical chaperone proteins that cooperate with ATP-dependent proteases during proteolysis, intrinsic chaperone-like properties have been assigned to some ATP-dependent proteases themselves which may be crucial for the degradation of non-native polypeptides (9, 10).

* This work was supported by Grant 96-48376 from the Russian Foundation for Basic Research and Grant 170516 from the Volkswagenstiftung (to V. N. L.) and by Grants La918/1-2 and SFB 184/B21 from the Deutsche Forschungsgemeinschaft (to T. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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which acts as a nucleotide exchange factor for Ssc1p. Thus, the mHsp70 system can cooperate with different ATP-dependent proteases in the degradation of non-native polypeptides in mitochondria.

EXPERIMENTAL PROCEDURES

Construction of pSu9(112)-P450scc—Recombinant DNA techniques were applied as described previously (37, 38). To generate pSu9(112)-P450scc, a DNA fragment encoding mouse dihydrololate reductase was excised from Su9(112)-dihydrololate reductase cloned in pGEM4 (39) by restriction digest with BamHI and HindIII. The HindIII site was filled in with Klenow and the vector was ligated with a BamHI-SmaI fragment of the plasmid pYeDP/coxIV-P450scc (40) encoding mature bovine P450scc lacking the N-terminal 75 residues. The resulting hybrid protein consisted of the 112 N-terminal amino acids of the F$_2$-ATPase subunit 9 fused to truncated P450scc.

Yeast Strains and Growth Conditions—Yeast strains carrying mutations in PIM1 were previously described (26). To establish the dependence on the proteolytic activity, PIM1 and a proteolytically inactive variant, $\text{PIM}_1^{	ext{S1015A}}$, were expressed in $\text{spin}1$ cells from a multicytoplasmid under the control of a galactose-inducible promoter (26). Cells were grown at 30 °C on selective medium containing 2% glucose and 0.5% galactose to induce expression from the GAL1 promoter. Wild type and mutant PIM1 protease were approximately 30-fold overexpressed in these cells (26). Yeast strains carrying mutations in YTA10 or YTA12 are derivatives of W303-1A and are described elsewhere (28). Yta10 or yta12 mutant strains were grown on YP medium supplemented with 2% galactose and 0.5% lactate at 30 °C according to standard procedures. Yeast strains carrying the temperature-sensitive mutant allele $\text{ssc}1-3$ (41) or $\text{mgI}1-3$ (42) were grown at 24 °C on selective medium containing 2% galactose and 0.5% lactate. The $\text{mdj}1-7$ mutant strain (43) was grown at 24 °C on lactate medium containing galactose instead of glucose.

Import of Preproteins into Isolated Mitochondria and Protein Degradation—Mitochondria were isolated as described previously except that zymolyase treatment was performed at 24 °C in the case of temperature-sensitive strains (44, 45). P450scc and pSu9(112)-P450scc were transcribed using SP6 polymerase and synthesized in rabbit reticulocyte lysate in the presence of [$^{35}$S]methionine (Promega) according to published procedures (46). Import of the radiolabeled preproteins was performed for 25 min at 25 °C essentially as described (15) and halted by the addition of vinaminycin (2 μM). To allow for proteolysis of newly imported proteins, mitochondria were further incubated in import buffer at 37 °C. At the time points indicated, aliquots were withdrawn and diluted 3-fold with ice-cold SHKCI (50 mM HEPES/KOH, pH 7.4, 0.6 mM sorbitol, 80 mM KCl). To digest non-imported precursor proteins, samples were treated with trypsin (75 μg/ml) for 20 min at 4 °C when indicated. Protease digestion was inhibited by adding a 20-fold excess (w/v) of soybean trypsin inhibitor. Mitochondria were then reisolated by centrifugation, washed with SHKCI containing 1 mM phenylmethylsulfonyl fluoride and separated into soluble and pellet fractions by centrifugation as described previously (36). Protein fractions were analyzed by SDS-PAGE, followed by autoradiography and immunoblotting using polyclonal antisera directed against cytochrome $\text{b}_2$ (cyt $\text{b}_2$), the ADP/ATP carrier (AAC), and Mge1p. T, total; P, pellet fraction; S, supernatant fraction. B, digitonin fractionation of mitochondria. After import of pP450scc and trypsin digestion, mitochondria were treated with increasing amounts of digitonin in the presence of proteinase K. After centrifugation, proteins in the pellet fraction were analyzed by SDS-PAGE and blotted onto nitrocellulose. Immunoblotting of the endogenous marker proteins cytochrome $\text{b}_2$ (Cyt $\text{b}_2$) and Mge1p (■) was performed using a chemiluminescence detection kit and quantified by laser densitometry. The amount of the protein in samples incubated with proteinase K in the absence of digitonin was set to 100%. ▲, pP450scc, precursor form of P450scc; △, mP450scc, mature form of P450scc.

RESULTS

Bovine Apocytochrome P450scc Is Imported into Yeast Mitochondria, but Is Not Correctly Sorted to the Inner Membrane—The precursor of bovine apocytochrome P450scc (pP450scc) was synthesized in rabbit reticulocyte lysate in the presence of [$^{35}$S]methionine and imported into isolated yeast mitochondria for 25 min at 25 °C, and non-imported preproteins were digested with trypsin as described under “Experimental Procedures.” Mitochondria were then resuspended in 0.1 M Na$_2$CO$_3$, pH 11.5, and 1 mM phenylmethylsulfonyl fluoride and separated into soluble and pellet fractions. Mitochondria were further incubated in import buffer at 37 °C. At the time points indicated, aliquots were withdrawn and diluted 3-fold with ice-cold SHKCI (50 mM HEPES/KOH, pH 7.4, 0.6 mM sorbitol, 80 mM KCl). To digest non-imported precursor proteins, samples were treated with trypsin (75 μg/ml) for 20 min at 4 °C when indicated. Protease digestion was inhibited by adding a 20-fold excess (w/v) of soybean trypsin inhibitor. Mitochondria were then reisolated by centrifugation, washed with SHKCI containing 1 mM phenylmethylsulfonyl fluoride and separated into soluble and pellet fractions by centrifugation as described previously (36). Protein fractions were analyzed by SDS-PAGE, followed by autoradiography and immunoblotting using polyclonal antisera directed against cytochrome $\text{b}_2$ (cyt $\text{b}_2$), the ADP/ATP carrier (AAC), and Mge1p. T, total; P, pellet fraction; S, supernatant fraction. B, digitonin fractionation of mitochondria. After import of pP450scc and trypsin digestion, mitochondria were treated with increasing amounts of digitonin in the presence of proteinase K. After centrifugation, proteins in the pellet fraction were analyzed by SDS-PAGE and blotted onto nitrocellulose. Immunoblotting of the endogenous marker proteins cytochrome $\text{b}_2$ (Cyt $\text{b}_2$) and Mge1p (■) was performed using a chemiluminescence detection kit and quantified by laser densitometry. The amount of the protein in samples incubated with proteinase K in the absence of digitonin was set to 100%. ▲, pP450scc, precursor form of P450scc; △, mP450scc, mature form of P450scc.

Mitochondria were treated with carbonate at pH 11.5. This treatment results in the release of soluble proteins into the supernatant fraction, while integral membrane proteins, such as the ADP/ATP carrier, are resistant toward extraction under these conditions. P450scc was almost exclusively recovered from the soluble fraction upon alkaline extraction of the mitochondria, similar to cytochrome $\text{b}_2$ and Mge1p, soluble proteins of the intermembrane and matrix space, respectively (Fig. 1A). Apparently, bovine P450scc is not inserted into the inner membrane of yeast mitochondria. To localize P450scc, yeast mitochondria were treated with increasing amounts of digitonin in the presence of externally added protease. The intermembrane and matrix space became successively accessible to the protease as indicated by the marker proteins cytochrome $\text{b}_2$ (Cyt $\text{b}_2$) and Mge1p (■) (Fig. 1B). Newly imported precursor and mature P450scc were released from mitochondria in parallel with Mge1p indicating that both forms are localized in the mitochondrial matrix (Fig. 1B). These results demonstrate that bovine apocytochrome P450scc is imported into yeast mitochondria and, although

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid.
with low efficiency, processed to the mature form. In contrast to adrenocortical mitochondria, however, P450scc molecules are not inserted into the inner membrane but accumulate in the matrix space. Thus, intramitochondrial sorting appears to depend on a specific sorting pathway in adrenocortical mitochondria or on the lipid composition of the membrane. It should be noted, however, that at least a fraction of P450scc appears to be correctly inserted into the inner mitochondrial membrane after expression in Saccharomyces cerevisiae, as submitochondrial particles exhibited cholesterol hydroxylase activity in the presence of externally added adrenodoxin and adrenodoxin reductase (40).

**ATP-dependent Degradation of P450scc by PIM1 Protease in the Matrix—**The stability of newly imported, missorted apocytochrome P450scc was analyzed in further experiments. After import of P450scc, the electrochemical gradient across the mitochondrial inner membrane was dissipated by adding the uncoupler valinomycin, and then mitochondria were incubated at 37 °C. Newly imported P450scc was degraded in mitochondria in a time-dependent fashion (Fig. 2). Proteolysis was impaired upon depletion of mitochondrial matrix ATP (Fig. 2).

To identify the protease responsible for the proteolytic breakdown of P450scc, mitochondria were isolated from yeast strains carrying mutations in either the PIM1 or the m-AAA protease, and the stability of newly imported P450scc was examined. Inactivation of either protease does not affect the accumulation of the other protease in mitochondria excluding indirect effects of the mutations (data not shown). Proteolysis of P450scc was impaired in mitochondria lacking PIM1 protease (Fig. 3A). Impairment of proteolysis of P450scc might result from a disturbed energy metabolism in pim1 mutant cells, which accumulate lesions in mtDNA and therefore are respiratory-deficient (24, 25). To exclude this possibility, the stability of P450scc was examined in Δpim1 mitochondria which lack mtDNA but contain PIM1 protease. Wild type and the proteolytically inactive variant PIM1S1015A protease carrying a mutation in the proteolytic center (26) were expressed in Δpim1 cells. Newly imported P450scc was degraded, although with reduced efficiency, in Δpim1 mitochondria harboring the wild type form of the protease, while proteolysis was not restored upon expression of PIM1S1015A protease (Fig. 3A). Thus, degradation of P450scc depends on the proteolytic activity of PIM1. In contrast, degradation of P450scc was not significantly affected by mutations in the catalytic sites of Yta10p (E559Q) or Yta12p (E614Q), which are subunits of the membrane-bound m-AAA protease (Fig. 3B). Both mutations have previously been observed to abolish the proteolytic breakdown of non-assembled polypeptides in the inner membrane by the m-AAA protease (27, 28). We conclude from these experiments that missorted P450scc is degraded by PIM1 protease in the mitochondrial matrix space.

**Missorted P450scc Is Prone to Aggregation in Mitochondria—**Newly imported P450scc, missorted to the mitochondrial matrix, most likely cannot attain its native conformation. It is therefore conceivable that P450scc is prone to aggregation in yeast mitochondria. P450scc, however, was recovered in the soluble fraction of mitochondria, even when these were incubated at 37 °C for 15 min (Fig. 4). Likewise, the solubility of P450scc was not affected after import into mitochondria lacking Yta10p or Yta12p and therefore m-AAA protease activity (Fig. 4). In contrast, P450scc formed insoluble aggregates in mitochondria lacking PIM1 protease (Fig. 4). These results demonstrate that missorted P450scc is prone to aggregation in yeast mitochondria and further substantiate the requirement of PIM1 protease for the proteolytic breakdown of P450scc.

The aggregation of P450scc in the absence of PIM1 might reflect a deficiency in a chaperone-like function of the protease, which has recently been proposed to exist independent of its proteolytic activity (50). Therefore, proteolytically inactive PIM1S1015A was overexpressed in pim1 null mutant cells, and aggregation of newly imported P450scc was analyzed (Fig. 4). Replacement of the conserved serine 1015 by alanine abolishes proteolytic activity of PIM1 but does not affect the overall protein stability nor the ATP-dependent assembly of the pro-
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...and mature form of P450scc was observed in all strains. P450scc prior to incubation at 37 °C. Similar aggregation of the precursor and mature form of P450scc was observed in all strains.

Requirement of the mtHsp70 System for the PIM1-mediated Degradation of P450scc—To examine a possible role of the mitochondrial Hsp70 protein Ssc1p in preventing aggregation of P450scc, we used mitochondria from the ssc1–3 yeast strain containing a temperature-sensitive mutant form of Ssc1p (41). Furthermore, the stability of P450scc was analyzed in mitochondria carrying a conditional mutant form of Mdj1p (mdj1–7) (43), which also seems to act as a molecular chaperone in mitochondria and ensures efficient binding to and release from Ssc1p (15, 51). After import of pP450scc into mitochondria at permissive temperature, samples were incubated at 37 °C to inactivate the mutant forms of the proteins and to allow degradation to occur. While P450scc was degraded in wild type mitochondria, proteolysis was impaired in ssc1–3 and mdj1–7 mutant mitochondria at non-permissive temperatures (Fig. 5A). P450scc formed insoluble aggregates under these conditions (Fig. 5B). Thus, similar to other misfolded proteins, the degradation of P450scc depends on Ssc1p and Mdj1p which stabilize P450scc molecules against aggregation and thereby allow their proteolytic breakdown by PIM1.

Polypeptide binding to Ssc1p is also regulated by Mge1p, which acts as a nucleotide exchange factor for Ssc1p (42, 52–54). A possible role of Mge1p during proteolysis of misfolded polypeptides in mitochondria, however, has not been reported until now. The degradation of P450scc was therefore analyzed in mitochondria carrying the temperature-sensitive mutant allele mge1–3 (42). Inactivation of Mge1p–5p at non-permissive temperature resulted in the stabilization of newly imported P450scc (Fig. 5A). In contrast to ssc1–3 or mdj1–7 mitochondria, P450scc accumulated in a soluble form in mge1–3 mitochondria (Fig. 5B), most likely due to an impaired release of P450scc from Ssc1p (52). These results establish a crucial function of all components of the mtHsp70 system during the PIM1-mediated proteolysis of misfolded proteins in mitochondria.

An Apo cytchrome $P_450$scs Variant with an Amino-terminal Transmembrane Region Is Anchored to the Inner Membrane—To ensure sorting of bovine apocytochrome $P_450$scs to the mitochondrial inner membrane, the targeting sequence and the amino-terminal 75 amino acid residues of P450scc were replaced by the first 112 amino acids of the subunit 9 of the $F_0$--$F_1$-ATPase of Neurospora crassa. This sequence contains the mitochondrial targeting sequence and the first transmembrane peptide of the outer membrane nor degraded by protease which was externally added to mitoplasts suggesting its presence in an internal mitochondrial compartment (Fig. 6B). The association of $S u_9$ (112)-P450scc with the inner membrane was examined by treatment of mitochondria with carbonate at pH 11.5 (Fig. 6B). In contrast to P450scc, the majority of newly imported $S u_9$ (112)-P450scc was recovered from the pellet fraction after extraction of mitochondria indicating the insertion of the protein into the inner membrane. In agreement with these findings, $S u_9$ (112)-P450scc became solubilized in parallel with the ADP/ATP carrier when mitochondria were treated with increasing amounts of digitonin (data not shown). We conclude from these experiments that $S u_9$ (112)-P450scc is tightly associated with the inner membrane.

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**FIG. 6. Import and localization of Su9(112)-P450sccc in yeast mitochondria.** A, Su9(112)-P450sccc is imported into mitochondria in a Δψ-dependent manner. Newly synthesized Su9(112)-P450sccc was imported into isolated mitochondria for 20 min at 25 °C in the presence (−Δψ) or absence (+Δψ) of valinomycin (0.5 μM), oligomycin (20 μM), and antimycin A (8 μM). Non-imported preproteins were digested with trypsin when indicated, p, precursor form of Su9(112)-P450sccc; m, mature form of Su9(112)-P450sccc. B, newly imported Su9(112)-P450sccc is sorted to the mitochondrial inner membrane. After import of Su9(112)-P450sccc for 20 min at 25 °C, the outer membrane was disrupted by osmotic swelling in the presence or absence of proteinase K (20 μg/ml/PK) as indicated. In parallel, mitochondria were extracted with 0.1 M Na₂CO₃, pH 11.5, and split into soluble and pellet fractions by centrifugation as in Fig. 1. Proteins in both fractions were separated by SDS-PAGE, blotted onto nitrocellulose, and analyzed by autoradiography and immunoblotting using cytochrome b₅ (cyt b₅), the ADP/ATP carrier (AAC), and Mge1p as marker proteins.

**FIG. 7. Degradation of Su9(112)-P450sccc in the mitochondrial inner membrane is mediated by the m-AAA protease.** A, the stability of Su9(112)-P450sccc in mitochondria containing proteolytically inactive Yta10p and Yta12p. Su9(112)-P450sccc was imported for 20 min at 25 °C into mitochondria isolated from wild type cells (WT●), from a Δyta10 strain expressing YTA10/S508Q (yta10/S508Q)[□], and from a Δyta12 strain expressing YTA12/S614Q (yta12/S614Q)[□] as described under "Experimental Procedures." Proteolysis of newly imported Su9(112)-P450sccc at 37 °C was determined as in Fig. 3. B, degradation of Su9(112)-P450sccc in the presence of proteolytically inactive PIM1 protease. Su9(112)-P450sccc was imported into Δpim1 mutant mitochondria harboring wild type PIM1 protease (pim1/PIM1)[■] or the proteolytically inactive PIM1/S505A protease (pim1/S505A)[●], and the stability at 37 °C was determined as described in Fig. 3.

Su9(112)-P450sccc into mitochondria and its insertion into the inner membrane. The stability of newly imported, membrane-associated Su9(112)-P450sccc was then examined at non-permissive temperature (Fig. 8). While Su9(112)-P450sccc was degraded in wild type mitochondria, proteolysis was impaired upon inactivation of Ssc1p, Mdj1p, or Mge1p in ssc1–3, mdj1–7, or mge1–3 mitochondria, respectively. These results demonstrate that degradation of membrane-bound Su9(112)-P450sccc by the m-AAA protease depends on Ssc1p and the co-chaperones Mdj1p and Mge1p which act after insertion of the polypeptide into the inner membrane.

**DISCUSSION**

Molecular chaperone proteins fulfill essential functions during the proteolysis of misfolded polypeptides in mitochondria. The mtHsp70 system cooperates not only with PIM1 protease in the degradation of non-native polypeptides accumulating in the matrix, but in addition is required for the proteolytic breakdown of at least some polypeptides which are associated with the inner membrane and degraded by the m-AAA protease. The proteolysis of misfolded polypeptides depends on the activities of Ssc1p and Mdj1p, which both exert chaperone activity and thereby most likely prevent the irreversible misfolding of polypeptides prone to degradation. Moreover, we assign a crucial role for the proteolysis of misfolded proteins to Mge1p, which acts as a nucleotide exchange factor of Ssc1p.
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P450scc was imported into wild type (WT) mge1–3 degradation of at least some membrane-associated polypeptides from the lipid bilayer. Nevertheless, activity of the protease might be needed to stabilize polypeptide proteins which are prone to degradation suggesting tides, a prerequisite for their degradation. Mge1p inhibits the release of associated substrate polypeptides dissociate from Hsp70 proteins (61–63). Thus, ATP to the chaperone (52–54). Upon ATP binding, substrate promotes the release of ADP from Ssc1p allowing the binding of they accumulate in the soluble state in the absence of Mge1p misfolded proteins by different proteases in mitochondria, most membrane-associated polypeptides, while proteolysis of other membrane proteins by the m-AAA protease is independently of the mtHsp70 system. Indeed, the proteolytic breakdown by the m-AAA protease of non-assembled, mitochondrially encoded subunits of respiratory chain complexes, which lack large solvent-exposed domains, was not affected in ssc1–3 mutant mitochondria at non-permissive temperature. Our results demonstrate an overlapping substrate specificity of the m-AAA protease with the PIM1 protease. A misfolded polypeptide is degraded by either protease depending on its localization in mitochondria. This finding is in agreement with genetic evidence indicating functional similarities between both proteases (50, 68). Next to nothing is known about the sequence specificity of ATP-dependent proteases in mitochondria. Studies on the E. coli Lon protease, however, suggest a rather degenerate specificity of Lon-like proteases (69). Similarly, the analysis of proteolytic fragments of a model protein generated by the i- and m-AAA protease did not reveal a conserved cleavage motif (35). The degradation of mitochondrial proteases by ATP-dependent proteases is apparently mainly initiated by a non-native conformation of the polypeptide. Molecular chaperones and chaperone-like properties of the proteases may therefore play a crucial role in the recognition of substrate polypeptides.

Acknowledgments—We are grateful to Dr. M. Waterman (University of Texas) for the bovine cytochrome P450scc cDNA clone, to Dr. S. A. Usanov (Minsk) for purified bovine cytochrome P450scc, and to Dr. E. Craig for the ssc1–3 mutant strain. The excellent technical assistance of Petra Robisch and Alexandra Weinzierl is gratefully acknowledged.

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