The ClpB Homolog Hsp78 Is Required for the Efficient Degradation of Proteins in the Mitochondrial Matrix*

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Molecular chaperones perform vital functions in mitochondrial protein import and folding. In yeast mitochondria, two members of the Clp/Hsp100 chaperone family, Hsp78 and Mcx1, have been identified as homologs of the bacterial proteins ClpB and ClpX, respectively. In this report we employed a novel quantitative assay system to assess the role of Hsp78 and Mcx1 in protein degradation within the matrix. Mitochondria were preloaded with large amounts of two purified recombinant reporter proteins exhibiting different folding stabilities. Proteolysis of the imported substrate proteins depended on the mitochondrial level of ATP and was mediated by the matrix protease Pim1/LON. Degradation rates were found to be independent of the folding stability of the reporter proteins. Mitochondria from hsp78Δ cells exhibited a significant defect in the degradation efficiency of both substrates even at low temperature whereas mcx1Δ mitochondria showed wild-type activity. The proteolysis defect in hsp78Δ mitochondria was independent from the aggregation behavior of the substrate proteins. We conclude that Hsp78 is a genuine component of the mitochondrial proteolysis system required for the efficient degradation of substrate proteins in the matrix.

All prokaryotic and eukaryotic organisms express several chaperone proteins of the Clp/Hsp100 family (1, 2). Members of this chaperone family were first described in Echerichia coli as ATP-dependent partner proteins of the serine-type protease ClpP. Apart from their function as cofactors of the protease, proteins of the Clp/Hsp100 family are involved in a variety of cellular activities due to their ability to influence changes in the folding and assembly state of proteins (3). Bacteria contain various subtypes of the Clp/Hsp100 chaperone proteins, whereas only three family members have been identified in Saccharomyces cerevisiae. Hsp104, which is located in the cytosol (4), and Hsp78, found in the mitochondrial matrix (5), are homologs of the bacterial ClpB and therefore belong to a subtype that shows no direct association with the ClpP protease (6). ClpB has recently been shown to be involved in the reaction of aggregated proteins in cooperation with the Hsp70 system in E. coli (7–9). Since a similar activity had previously been described for the cytosolic Hsp104 (10, 11), the current view is that proteins of the ClpB subtype are mainly required for the protection of cellular protein function under severe stress conditions (7, 12, 13). Evidently, cellular proteins can be solubilized from heat-induced aggregates by ClpB-type chaperones and are released in a state that is recognized and stabilized by the Hsp70 system, which promotes further folding to the native conformation (8, 11, 14). A similar role in thermotolerance and stress protection has been suggested for the mitochondrial homolog Hsp78 (15, 16). In the absence of functional Hsp78, cells are viable but, under extreme heat stress, hsp78Δ mitochondria lose their respiratory function. Recent in vitro experiments suggested that Hsp78 can reanimate denatured polypeptides in an ATP-dependent reaction in cooperation with the mitochondrial Hsp70 system (17). The third Clp/Hsp100 homolog in S. cerevisiae, Mcx1 (for mitochondrial ClpX), is also localized in the mitochondrial matrix. Being related to the bacterial ClpX protein, it belongs to a different subtype of Clp/Hsp100 chaperones than Hsp78 (18). In E. coli, ClpX forms a stable complex with ClpP and is thought to determine the substrate specificity of the protease (19), acting as an ATP-dependent unfoldase that prepares substrates for degradation. Interestingly, in contrast to higher eukaryotes, there is no ClpP protease in yeast mitochondria, and the function of Mcx1 remains unclear.

The involvement of Clp/Hsp100 proteins in degradative proteolytic reactions in bacteria suggests a similar role for the related proteins in eukaryotic organelles. However, the involvement of mitochondrial chaperone proteins as factors determining protein turnover are so far not well understood. Mitochondrial protein degradation requires ATP hydrolysis (20) and is largely mediated by two proteolytic systems, the soluble ring-shaped Pim1/LON complex (for proteolysis in mitochondria), found in the matrix, and two inner membrane-bound AAA proteases, which mainly degrade proteins integrated into the mitochondrial inner membrane (21). Most identified substrates of Pim1 are soluble matrix proteins, yet some substrate overlap may exist with the membrane proteases (22, 23). Pim1 is not essential for cell survival but is required for the maintenance of an intact mitochondrial genome (24). The induction of Pim1 expression upon heat stress may be indicative of a role in the degradation of denatured and misfolded proteins. Efficient degradation of misfolded proteins by Pim1 required a functional cooperation with the mitochondrial Hsp70, Sac1, and its co-chaperone Djl1, which hold aggregation-prone polypeptides in a soluble form (25). Interestingly, in addition to their proteolytic activities, chaperone properties have been attributed to Pim1 as well as to the components of the membrane-bound proteases (22, 26), which suggests that chaperone-like recognition of non-native polypeptides could be a prerequisite for efficient protein degradation.

For this report we employed a novel assay system with...
imported recombinant substrate proteins to study protein degradation in the mitochondrial matrix, as well as the involvement of Clp/Hsp100 chaperones in this process. In contrast to previous mitochondrial degradation assays using radiolabeled preproteins in small amounts, we challenged the proteolytic system with large amounts of substrate proteins (27), an assay system that allowed us to determine the kinetics of the degradation reaction. Two reporter proteins with different folding stabilities were accumulated in the mitochondrial matrix to compare the energy requirement of their degradation and to examine the involvement of the chaperones Hsp78 and Mcm1 in the process. We found that the folding state of the substrates did not affect the ATP requirement of the Pim1-mediated degradation reaction. Surprisingly, the mitochondrial Hsp100 chaperone Hsp78 was required for efficient degradation of imported substrate, whereas the second mitochondrial Hsp100 family member, Mcm1, did not influence the efficiency of protein degradation. Because proteins of the ClpB family have mainly been implicated in the reactivation of aggregated polypeptides, the involvement of Hsp78 in proteolysis represents a new functional aspect of this chaperone class.

EXPERIMENTAL PROCEDURES

Yeast Strains and Isolation of Mitochondria—S. cerevisiae strains used in this study are summarized in Table I. To generate isogenic Clp/Hsp100 mutant strains, strains SAL3 (his3Δ, S42C, and N49C) (33) were crossed using standard methods of yeast genetics. By tetrad dissection, the single mutants, WYVY40 (his3Δ, SAL3) and WYV39 (mex1Δ), as well as the corresponding wild-type, WYV38 (WT), were isolated. The complete open reading frame of PIM1 was disrupted in the wild-type strain YPH499 (28) by transforming the HIS3 marker from plasmid pFA6-AHis3MX6 (29), which was amplified using oligonucleotides complementary to 100 bp upstream and downstream of the PIM1 open reading frame, resulting in the rho- strain KRY01 (pim1Δ1). To create the corresponding rho+ wild-type strain to KRY02 (pim1Δ2), strain YPH499 was exposed to ethidium bromide (30), resulting in KRY01 WT (Fig. 3). Mitochondria were isolated from these strains according to published procedures (31) from yeast cultures grown at 30 °C in YP medium (1% yeast extract, 2% peptone) containing 3% glycerol, or 2% glucose in the case of respiration-deficient cells.

Purification of Preproteins and Import Assay—Recombinant cytochrome b2-DHFR preproteins were expressed in E. coli BMH71-18 cells from plasmid pUE5577 (b2 (167), DHFR) (32) under the control of an IPTG-inducible promoter. For the construction of b2-DHFRΔ, three point mutations were introduced by site-directed mutagenesis, which led to amino acid changes in three positions (C7S, S42C, and N49C) (33). Preproteins were purified from inclusion bodies by several extractions with increasing urea concentrations according to a protocol modified from Schmid et al. (34). For the in vitro import reactions, saturating amounts of preproteins denatured in urea buffer (7 M urea, 30 mM MOPS (3-N-morpholino)propane sulfonic acid pH 7.2, 1 mM dithiothreitol) were incubated with isolated mitochondria as previously published (27) to a final concentration of 250 pmol of preproteins per mitochondrion. Unless stated otherwise, the reporter proteins were detected after SDS-PAGE and Western blotting by immunodecoration with an affinity-puriﬁed antisera against mouse DHFR. DHFR signals were detected with the Enhanced Chemiluminescence system (Amersham Biosciences) and quantiﬁed using Image Master 1D software (Amersham Biosciences) and EXCEL 98 (Microsoft).

Results

Assay of the Folding State of DHFR in Vivo—After import of b2-DHFR or b2-DHFRΔ for 15 min at 25 °C, mitochondria were resolated for 2 min in the presence of 50 μg/ml ice-cold trypsin to remove excess preprotein. The trypsin-containing supernatant was removed completely. 25 μg of mitochondria were lysed in 100 μl of lysis buffer (0.3% Triton X-100, 30 mM Tris pH 7.4, 5 mM EDTA, 100 mM KCl) containing 100 μg/ml proteinase K (PK). After incubation on ice for 5 min, PK was inactivated by addition of 3 mM phenylmethylsulfonyl fluoride (PMSF). Samples were precipitated with trichloroacetic acid and analyzed as described.

Degradation Assay—Recombinant b2-DHFR preproteins were imported into 150 μg of isolated mitochondria for 15 min at 25 °C as described. Subsequently 1/10 of the reaction mix was taken as an import sample. A second import sample corresponding to 1/10 of the original mix was taken after PK treatment (100 μg/ml) for 10 min on ice. After resolation, mitochondria were resuspended in 800 μl of prewarmed import buffer (250 mM sucrose, 10 mM MOPS-KOH pH 7.2, 80 mM KCl, 5 mM MgCl2) supplied with 4 mM NADH, and either 10 units/ml apyrase and 0.1 mM oligomycin, or an ATP-regenerating system (3 mM ATP, 20 mM creatine phosphate, 200 μg/ml creatine kinase), and incubated at 25 °C or 37 °C. At time points 0, 15, 30, 60, 120, 180, and 240 min, 100-μl aliquots were taken, mitochondria were resolated, and the amount of remaining reporter protein was determined as described.

Aggregation Assay—Following import of b2-DHFR at 25 °C, mitochondria were resolated and incubated for 15 min at 25 °C or 37 °C in fresh import buffer containing ATP. 30 μg of mitochondria were treated with 100 μg/ml PK for 10 min on ice, and samples were split into an import control and a sample for the aggregation assay. After resolation and washing with SE buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH pH 7.2), mitochondria were lysed on ice in 200 μl lysis buffer (0.3% Triton X-100, 30 mM Tris-HCl pH 7.4, 80 mM KCl, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 3 mM ATP, 5 mM MgCl2, and 10 μM Oligomycin) and incubated at 25 °C for 10 min. At time point 0, 15, 30, 60, 120, 180, and 240 min, 100-μl aliquots were taken, mitochondria were resolated, and the amount of remaining reporter protein was determined as described.

RESULTS

Import and Folding State of Recombinant b2-DHFR Reporter Proteins in the Mitochondrial Matrix—To determine the factors that influence protein degradation rates in the mitochondrial matrix, we surveyed the turnover of two types of reporter proteins. Both were composed of the amino-terminal part of the mitochondrial targeting signal, fused to the entire mouse dihydrofolate reductase cytochrome b2, providing the mitochondrial targeting signal, fused to the entire mouse dihydrofolate reductase (DHFR) (Fig. 1A). By a deletion of 19 amino acids in the prescission of cytochrome b2, the insoluble fraction was re-extracted with lysis buffer and centrifuged again at 100,000 × g for 30 min. All samples were analyzed by SDS-PAGE, Western blotting, and immunodetection of DHFR as described.

The precursor proteins were expressed and purified as recombinant proteins from E. coli cells and imported in vitro into isolated mitochondria from S. cerevisiae (27). To obtain maximal import efficiency, the recombinant precursor proteins were...
denatured in buffer containing urea before the import reaction. The imported reporter proteins were detected by Western blot and immunodecoration with an antibody directed against the DHFR moiety. Import into wild-type mitochondria was efficient and specific as shown by the processing to the mature form and the dependence on a mitochondrial membrane potential (Fig. 1B, lanes 1 and 2, upper and lower panels). The processed forms were protected from externally added proteases, confirming their mitochondrial localization (Fig. 1B, lane 7, upper and lower panels). Efficient import of both reporter proteins was also observed in mitochondria isolated from strains lacking the mitochondrial Clp/Hsp100 family members Hsp78 or Mcx1 (Fig. 1B, lanes 3–6 and 9–12, upper and lower panels).

Using the very efficient in vitro import system with recombinant preproteins, we were able to accumulate large amounts of potential substrate proteins in the mitochondrial matrix. By comparison with standardized Western blots, we estimated that 50–80 pmol of the b2-DHFR reporter proteins per mg of mitochondria were imported. Therefore, the proteolytic machinery in the matrix was challenged with large amounts of substrate proteins in the assay system. We first tested whether the mitochondrial chaperone system was able to fold the accumulated proteins to a stable conformation. We analyzed the folding state of the DHFR domains of b2-DHFR and b2-DHFRds, preproteins for 15 min at 25 °C in the presence or absence of a membrane potential across the inner mitochondrial membrane (Δψ). The precursor proteins (p) and their proteinase K (PK)-protected processed forms (i) were detected after Western blotting by antibodies against mouse DHFR. C, in vivo analysis of the DHFR folding state. Following import of b2-DHFR or b2-DHFRds, wild-type mitochondria (WVY38) were lysed on ice under non-denaturing conditions in the presence of PK. Precursor (p) and processed (i) forms of the fusion proteins, as well as protease-resistant folded DHFR (f), are immunodetected by antibodies against DHFR.
was therefore able to reach its native folding state. In contrast, the construct b\(_2\)-DHFR\(_{ds}\) with its destabilized DHFR domain was completely degraded under the same conditions (Fig. 1C, lane 5), indicating that it did not acquire a protease-resistant conformation.

\[\text{Both Reporter Proteins Are Efficiently Degraded in Mitochondria—Using the in vitro import assay, mitochondria were pre-loaded with substrates for the degradation machinery. After the import reaction was stopped and excess preprotein was removed by a protease treatment, the fate of the reporter proteins in the matrix was followed during an incubation at 25 °C over the course of 4 h (Fig. 2A). Although the imported b\(_2\)-DHFR was folded to the native conformation, it was degraded with high efficiency (Fig. 2B, upper panel). The degradation kinetics of the destabilized b\(_2\)-DHFR\(_{ds}\) proved to be very similar to the folded b\(_2\)-DHFR, suggesting that the degradation rate is independent of the folding state of the substrate (Fig. 2B, lower panel).} \]

After 60 to 90 min, most of the respective imported proteins were degraded. Further degradation occurred until the maximum incubation time, albeit with lower rates. In this assay we typically did not observe the generation of specific fragments of lower molecular weight, indicating that the substrate proteins were degraded to small fragments that could not be recognized by the DHFR antibody used for detection. As a control, we also analyzed the levels of endogenous proteins of various mitochondrial subcompartments. In contrast to the imported foreign reporter proteins, the levels of the soluble matrix protein Mge1 as well as the inner membrane-associated proteins Tim44 and F\(_1\)\(\gamma\)\(\delta\), and the outer membrane protein Tom40 were surveyed using specific antibodies against these proteins.

Mitochondrial Degradation of Imported Recombinant Proteins Requires ATP and Is mediated by Pim1—Both chaperone function and proteolytic reactions have been shown to depend on ATP hydrolysis. Here we tested whether the proteolysis of the imported recombinant substrate proteins depended on the level of ATP in the mitochondrial matrix and which protease was responsible for the observed degradation. The major ATP-dependent protease of the mitochondrial matrix is the Pim1/
FIG. 3. Degradation of imported \( b_2\)-DHFR in the matrix depends on ATP and is catalyzed by Pim1. A, after import of \( b_2\)-DHFR for 15 min at 25 °C, respiration-deficient wild-type mitochondria (KRY01) were incubated at 25 °C with either apyrase and oligomycin to deplete ATP (−ATP), or with an ATP-regenerating system (+ATP). At time points 0, 15, 30, 60, 120, 180, and 240 min (lanes 3–9), aliquots were taken and the amount of remaining \( b_2\)-DHFR was determined by immunodetection after SDS-PAGE and Western blotting. B, proteolytic breakdown of imported \( b_2\)-DHFR at 25 °C was analyzed in mitochondria isolated from a \( pim1\Delta\) strain (KRY02) in the absence or presence of ATP.

LON protease. We constructed a \( pim1\Delta\) yeast strain that did not contain the Pim1/LON protease. Because the mutant yeast cells had a defect in mitochondrial respiration, a comparable respiration-deficient wild-type strain was generated. When we specifically depleted the ATP levels in the matrix of mitochondria derived from this wild-type strain, we found that the imported \( b_2\)-DHFR construct remained stable over the course of 240 min (Fig. 3A, upper panel). In the presence of excess ATP, supplied by an ATP-regenerating system, degradation was fast and efficient, as shown by the decreasing amount of \( b_2\)-DHFR (Fig. 3A, lower panel). In all cases, similar degradation efficiencies between respiring and non-respiring mitochondrial preparations were observed, indicating that the respiration competence has no major influence on mitochondrial proteolysis. In addition, no difference was observed between \( b_2\)-DHFR and \( b_2\)-DHFR\textsubscript{res} in the ATP dependence of the degradation (data not shown), confirming the conclusion that the folding state of substrate proteins was not a major determinant of the degradation rate. The ATP dependence of the reaction, in addition to the observed stability of endogenous matrix proteins, indicates that the reduction of the DHFR signals in the degradation assay was caused by an unspecified process. To determine whether Pim1 was responsible for the observed degradation of the \( b_2\)-DHFR preproteins, we imported large amounts of recombinant \( b_2\)-DHFR precursors into the matrix space of \( pim1\Delta\) mitochondria. In the absence of ATP, no degradation occurred over the course of 240 min in mitochondria lacking Pim1 (Fig. 3B, upper panel), as observed before in wild-type organelles. Under conditions of excess ATP, the imported \( b_2\)-DHFR remained equally stable over the full incubation period (Fig. 3B, lower panel). The same result was observed for the destabilized construct \( b_2\)-DHFR\textsubscript{dest}, which remained stable in the absence of ATP as well as in energized Pim1-deficient mitochondria (data not shown). This demonstrates that both recombinant model proteins were hydrolyzed by Pim1 in an ATP-dependent reaction. By the complete lack of degradation in \( pim1\Delta\) mitochondria, we can exclude that other proteolytic activities were involved in this process.

Deletion of \( \textit{Mcx1} \) Does Not Affect Degradation of Imported Preproteins—Because mitochondria contain homologs of the bacterial Clp/Hsp100 proteins that have been shown to participate in various proteolytic reactions, we analyzed their involvement in the Pim1-mediated degradation of imported \( b_2\)-DHFR reporter proteins. The bacterial ClpP protein can function as a subunit of the ClpP protease, assisting in substrate binding and preparation for degradation, but no direct function has been established for the mitochondrial ClpX homolog Mcx1 so far. Hence, we analyzed a possible involvement of Mcx1 in mitochondrial proteolysis by performing degradation assays in a deletion mutant lacking Mcx1. We found that both precursor proteins were efficiently degraded in \( mcx1\Delta\) mitochondria with the same rates as in wild-type mitochondria (Fig. 4); thus, the ClpX homolog Mcx1 does not appear to be involved in the turnover of matrix-localized proteins.

\textit{Hsp78} Is Required for Efficient Degradation of Imported Preproteins—The main function described to date of the mitochondrial ClpB homolog Hsp78 is the involvement in the maintenance of mitochondrial thermostolerance. Here we employed the degradation assay with recombinant imported preproteins to test whether Hsp78 was also involved in mitochondrial proteolysis. In the wild-type control, \( b_2\)-DHFR was degraded efficiently while mitochondria isolated from cells lacking Hsp78 showed a pronounced defect in degradation (Fig. 5A, left diagram). Although the reporter protein was still degraded in the absence of Hsp78, the overall degradation efficiency was significantly reduced compared with that of the wild-type mitochondria. Up to 80% of the initially present reporter proteins were degraded in wild-type mitochondria after 4 h of incubation whereas in \( hsp78\Delta\) mitochondria only about 40% were degraded. Interestingly, only minor effects of the \( hsp78\Delta\) mutation on the initial rate of degradation were observed. We also tested whether the proteolytic breakdown of the destabilized construct \( b_2\)-DHFR\textsubscript{dest} depended on Hsp78 (Fig. 5A, right diagram). The kinetics of the degradation reaction of the folding-deficient \( b_2\)-DHFR\textsubscript{dest} in the wild-type were generally similar to the folded \( b_2\)-DHFR but showed a higher rate in the first part of the incubation period. In \( hsp78\Delta\) mitochondria, we again observed a strongly reduced degradation efficiency (Fig. 5A, right diagram). Only at very long incubation times was the proteolysis of the destabilized \( b_2\)-DHFR\textsubscript{dest} accelerating again in \( hsp78\Delta\) mitochondria, an effect that was not observed with the folded reporter protein. In summary, Hsp78 was required for the efficient degradation of both DHFR folding variants, indicating that the chaperone was involved in the degradation process irrespective of the folding state of the substrate. Because the function of Hsp78 has been shown to be particularly important at elevated temperatures, we also surveyed proteolysis of the \( b_2\)-DHFR reporter proteins at 37 °C. The proteolytic breakdown of \( b_2\)-DHFR in the wild-type was virtually unchanged, and proteolysis in \( hsp78\Delta\) mitochondria was significantly reduced as observed at 25 °C (Fig. 5B, left diagram). In contrast to the assay at 25 °C, the construct containing the mutated DHFR domain remained stable at 37 °C in \( hsp78\Delta\) as well as in wild-type mitochondria (Fig. 5B, right diagram). However, some degradation occurred in both strains at very long incubation times. Taken together, the results demonstrate that the ClpB homolog Hsp78 was required for the efficient ATP-dependent degradation of proteins located in the mitochondrial matrix.
Solubility of b2-DHFR Reporter Proteins in the Mitochondrial Matrix Is Temperature-dependent and Independent of Hsp78

The most prominent biochemical function described for ClpB in E. coli is its ability to assist in the resolubilization of aggregated proteins in cooperation with the Hsp70 system. A possible reason for the reduced degradation in the absence of the ClpB homolog Hsp78 might be that the imported reporter proteins show an increased tendency to aggregate. We therefore tested whether the large amounts of imported polypeptides remained soluble in the matrix and whether a heat treatment changed their solubility.

After import of b2-DHFR or b2-DHFRds, mitochondria were incubated at 25 °C or 37 °C and then lysed with detergents under non-denaturing conditions (Fig. 6A). The lysate was subjected to a high-speed centrifugation to pellet aggregated proteins. The pellet was re-extracted once with lysis buffer to remove any contaminating proteins. The supernatant contained the soluble fraction of the mitochondrial proteins including the inner membrane-associated protein Tim44 and the ATP synthase subunit F1β, as well as solubilized integral membrane proteins such as Tim23 and the ADP/ATP carrier (Fig. 6C and data not shown).

When we analyzed the pellet fraction for the presence of the imported reporter proteins, we found that both b2-DHFR and the mutated b2-DHFRds were completely soluble at 25 °C in wild-type as well as in hsp78Δ mitochondria (Fig. 6B, lanes 2 and 3). Because the solubility of the imported reporter proteins was the same in wild-type and hsp78Δ mitochondria, the degradation defect in hsp78Δ mitochondria could not be caused by increased protein aggregation. When we incubated mitochondria at 37 °C after the import reaction was completed, b2-DHFR remained soluble but the folding-compromised b2-DHFRds was found as aggregated protein in the pellet.

**Fig. 4.** Degradation efficiency is not impaired in mcx1Δ mitochondria. b2-DHFR (left diagram) or b2-DHFRds preproteins (right diagram) were accumulated for 15 min at 25 °C in the matrix of wild-type (WVY38) and mcx1Δ mitochondria (WVY39). Protein degradation was analyzed as described for Fig. 2B. The amount of reporter protein present at time point 0 min was set to 100% for wild-type (black squares and circles) and mcx1Δ mitochondria (gray squares and circles), and the decrease over time was measured in relation to this value. Diagrams show the means and standard errors of the means of at least four independent experiments.

**Fig. 5.** Efficient degradation of both b2-DHFR proteins depends on Hsp78. A, degradation of matrix-accumulated b2-DHFR (left diagram) or b2-DHFRds (right diagram) was monitored in wild-type (WVY38, black squares and circles) and hsp78Δ mitochondria (WVY40, gray squares and circles) for 240 min at 25 °C. The amount of reporter proteins was quantified as described in the legend of Fig. 4. B, after import of b2-DHFR or b2-DHFRds for 15 min at 25 °C and proteinase K treatment, proteolytic breakdown of the reporter proteins at 37 °C was examined and quantified in wild-type and hsp78Δ mitochondria as described.
Aggregation of imported b2-DHFR constructs is not increased in Hsp100-deficient mitochondria. A, scheme of the experimental procedure. B, after accumulation of large molar amounts of b2-DHFR or b2-DHFRds in wild-type (WVY38) and hsp78A mitochondria (WVY40) for 15 min at 25 °C and protease K treatment, mitochondria were reisolated and further incubated for 15 min at 25 or 37 °C. After lysis, aggregated proteins (P) were separated from the detergent-soluble fraction (S) by high-speed centrifugation for 30 min at 100,000 × g, and the solubility of DHFR reporter proteins was determined by immunodetection of DHFR after SDS-PAGE and Western transfer. C, to control lysis efficiency, the solubility of inner membrane-associated and integral membrane proteins was surveyed using specific antibodies against Tim44 and Tim23.

(Fig. 6B, lanes 5 and 6). Again, no difference was detected in protein solubility between wild-type and hsp78A mitochondria. Aggregation of the destabilized b2-DHFRds construct was also observed in organelles lacking Mcx1 or both Clp/Hsp100 family members (data not shown). The stability of b2-DHFRds in the degradation assay at 37 °C in both wild-type and hsp78A mitochondria was most likely caused by the quantitative aggregation of the destabilized construct at the elevated temperature. Taken together, the degradation defect in hsp78A organelles is not correlated with increased aggregation of the imported substrates in the matrix, indicating that the involvement of Hsp78 in mitochondrial protein degradation is a genuine functional property different from its role in aggregation reactions.

DISCUSSION

We have studied the proteolytic breakdown of large amounts of substrate proteins by preloading the matrix of isolated mitochondria with recombinant reporter proteins and monitoring the turnover of these substrates. In a preliminary estimate, we determined the amount of Pim1/LON complexes to be in the range of 0.5–2 pmol/mg mitochondrial protein (data not shown). In comparison, the amount of imported reporter protein reached up to 50–80 pmol/mg mitochondrial protein. The main advantage of our assay system is therefore the possibility to study the degradation system in the organelle under substrate-saturating conditions. As in previous mitochondrial degradation assays that used small amounts of radiolabeled substrate proteins (23, 25), the degradation of the heterologous reporter proteins proved to be ATP-dependent and was mediated by the matrix protease Pim1/LON. The large amounts of substrate proteins present in the matrix raised the question to which extent mitochondrial chaperones participate in the turnover of the proteins. Proteins of the Clp/Hsp100 family have been shown to be prominently involved in proteolysis in E. coli as subunits of the ClpP protease complex (19, 38, 39). Because mitochondria from S. cerevisiae do not seem to contain a homolog of the bacterial ClpP protease, the functional relation between mitochondrial Clp/Hsp100 chaperones and proteases has remained unclear. We now report the unexpected finding that the mitochondrial ClpX homolog Hsp78 is required for the efficient degradation of imported substrate proteins in the matrix space. Because Hsp78-dependent degradation in mitochondria was found to be mediated by the protease Pim1/LON, our data represent the first evidence of a functional cooperation of a Hsp100 chaperone with a protease other than ClpP.

So far, a cooperation between chaperones and the Pim1/LON protease has been established for the Hsp70 system (23, 25). A synergistic action between the bacterial Hsp70 DnaK and the LON protease has been demonstrated, although DnaK does not seem to be absolutely required for degradation (40). In mitochondria, mutants in Hsp70 or in cochaperones like Mj1 showed a strongly reduced proteolysis of imported radiolabeled proteins. In contrast to our results, however, mainly misfolded proteins were found to be degraded. It is assumed that Hsp70 binds and stabilizes imported substrate proteins that did not acquire a native conformation. After release from Hsp70, these polypeptides are degraded. Most probably, the function of Hsp70 in degradation is mechanistically different from the involvement of the Hsp100 chaperone Hsp78. The abundant Hsp70 acts as a general chaperone for newly imported matrix proteins at an early stage of mitochondrial protein biogenesis, whereas Hsp78 seems to perform a role at a later stage in the proteolytic process, directly preparing substrate proteins for degradation. This conclusion is supported by the observation that the deletion of Hsp78 did not significantly change the initial degradation rate but rather reduced the overall extent of the proteolysis reaction. Interestingly, a deletion of Mxc1, the mitochondrial homolog of the genuine protease subunit ClpX (18), had no effect on the proteolytic breakdown of imported recombinant substrates. Mitochondria of higher eukaryotes, on the other hand, contain the ClpP protease system in which the respective ClpX homolog acts as a cofactor for the protease (41, 42). These results indicate that mitochondrial ClpX proteins can indeed function in proteolysis. The function of the ClpX homolog Mxc1 in yeast mitochondria, however, remains elusive.

Because endogenous matrix proteins remained largely stable during the experimental incubation period, the imported reporter proteins seemed to be selectively recognized for degradation. Not much is known about the signals that target proteins in the organelle for degradation. It was observed that the functional state of a polypeptide is an important determinant of mitochondrial protein turnover. For example, non-assembled complex subunits of the respiratory chain and other non-native proteins synthesized in the mitochondrion are subject to rapid
hydrolysis (43). Newly imported polypeptides that fail to fold and assemble into a functional form are similarly unstable inside the organelle (25). Under stress conditions, such as elevated temperatures, it was observed that certain endogenous proteins were also degraded after import in vivo (44). It is likely that thermal denaturation rendered these polypeptides susceptible to proteolysis. However, an unfolded conformation may not be the major determinant for proteolytic breakdown in mitochondria, as we did not observe major differences in the degradation of the folded and the folding-compromised reporter protein. This indicates that the proteolysis of mitochondrial proteins is a very specific process in which substrates are identified not only by their conformational state but also by other unknown criteria. In contrast to previous observations (25), we were not able to detect specific proteolytic fragments in significant amounts. Because the polyclonal antisem that was used is able to recognize the entire DHFR domain, the absence of specific fragments indicates a processive and complete hydrolysis of the substrate. Interestingly, despite saturating amounts of substrate proteins, we were not able to detect a difference in the energy requirement of degradation between the folding-destabilized and the stably folded substrate. This finding suggested that the major ATP-consuming step was the hydrolysis of the polypeptide chain rather than an unfolding reaction that prepared the folded substrate for degradation. There are two possible reasons why no ATP dependence for unfolding was observed. Previous experiments proposed that Pim1/LON has an independent chaperone-like function in addition to its core protease activity (45, 46). It is feasible that protease and chaperone activities are functionally too closely coupled to distinguish a separate ATP requirement for substrate unfolding. Alternatively, the large amounts of imported protein may have overloaded the proteolytic system so that the hydrolysis of the degradation-competent polypeptide chain by Pim1 became the rate-limiting step rather than substrate unfolding.

Because the dependence of efficient proteolysis on Hsp78 was not caused by a possible unfolding activity of the chaperone, the question arises how the function of Hsp78 in thermotolerance is correlated with its function in protein degradation. Homologs of Hsp78, mainly ClpP in E. coli and Hsp104 in S. cerevisiae, have been implicated in reactions ensuring cell survival under extreme heat stress (4, 47). Under stress conditions, both proteins have been shown to protect substrate polypeptides from irreversible denaturation by aggregation. However, neither ClpP nor Hsp104 are able to prevent protein aggregation; instead, they were found to reverse the process by extracting proteins from aggregates (10, 48). For ClpP, a direct interaction with protein aggregates has been shown (8). This function may become vital when the level of protein denaturation exceeds the capacity of other chaperones, e.g. of the Hsp70 class, to prevent aggregation. In yeast, Hsp78 proved indispensable for the maintenance of mitochondrial function under heat stress. Again, the chaperone was incapable of preventing heat-induced inactivation of mitochondrial protein synthesis, yet translation reactions were only resumed when Hsp78 was present (49, 50). Recently, it was demonstrated that Hsp78 was capable of reactivating a denatured substrate in cooperation with the mitochondrial Hsp70 system in vitro (17). To assess the relative influence of Hsp78 on aggregation versus degradation processes, we analyzed the aggregation behavior of the imported reporter proteins in the mitochondrial matrix. In accordance with the observations in other cellular systems, we observed that the solubility of the imported proteins did not depend on the presence of Hsp78, indicating that the chaperone did not prevent aggregation. At normal temperatures, the efficiency of degradation of soluble substrates was significantly reduced by a deletion of Hsp78 under the same conditions. We conclude that the activity of the ClpP/Hsp100 chaperone in the Pim1-mediated degradation process under normal conditions did not involve the reactivation of insoluble polypeptides. At elevated temperatures, the destabilized reporter protein aggregated quantitatively. Aggregation was independent of the presence of Hsp78, and degradation was completely abolished in both the presence and the absence of Hsp78. Taken together, the results demonstrate that the requirement for Hsp78 in degradation did not correlate with the aggregation of the substrate proteins, as indicated by the fact that proteolysis in mitochondria lacking the ClpP/Hsp100 chaperone was also negatively affected in the absence of any aggregates. Expression of Hsp78 and Pim1 is induced upon heat treatment, and both proteins are required for mitochondrial function under heat stress. The physiological function of Hsp78 may be to support Pim1 in the removal of non-native polypeptides, a process that will be more eminent under protein-denaturing stress conditions.

The novel function of Hsp78 in a proteolytic process catalyzed by the protease Pim1/LON indicates that the cooperation of the mitochondrial chaperone and protease systems is not restricted to the Hsp70 system. The role of Hsp78 in mitochondrial degradation is distinct from its recently described function in the reactivation of insoluble proteins, and the cooperation between Hsp78 and Pim1/LON described here adds a novel aspect to our understanding of the chaperone-protease network in mitochondria.

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