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Loss of Mitochondrial hsp60 Function: Nonequivalent Effects on Matrix-Targeted and Intermembrane-Targeted Proteins

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We have created yeast strains in which the mitochondrial chaperonin, hsp60, can be either physically depleted or functionally inactivated. Cells completely depleted of hsp60 stop growing but retain for awhile the capacity to reaccumulate hsp60. While this newly made hsp60 is targeted to and processed correctly within the mitochondrion, assembly of a functional hsp60 complex does not occur. Rather, the hsp60 monomers are localized in different-size soluble complexes containing another mitochondrial chaperone, the mitochondrial form of hsp70. A number of other mitochondrial matrix-targeted proteins synthesized in the absence of functional hsp60 are imported into mitochondria but often show some buildup of precursor forms and, unlike hsp60, accumulate as insoluble aggregates. By contrast, several mitochondrial proteins normally targeted to the intermembrane space show normal processing in the complete absence of a functional hsp60 complex. Similar and complementary results were obtained when we examined the metabolism of matrix- and intermembrane space-localized proteins in cells expressing three different temperature-sensitive alleles of *HSP60*. In all cases, matrix-targeted proteins synthesized at nonpermissive (i.e., hsp60-inactivating) temperatures were correctly targeted to and processed within mitochondria but accumulated predominantly or totally as insoluble aggregates. The metabolism of two intermembrane space proteins, cytochrome b_2 and cytochrome c_1, was unaffected at the nonpermissive temperature, as judged by the correct processing and complete solubility of newly synthesized forms of both proteins. These findings are discussed with regard to current models of intermembrane targeting.

The *Saccharomyces cerevisiae* mitochondrial chaperonin, hsp60, is an essential gene product whose function is required for the proper formation of a number of mitochondrial enzyme complexes (6, 24). It has been shown for some mitochondrially targeted proteins that hsp60 interacts with and catalyzes the refolding of such polypeptides following their import into the mitochondrial matrix (21). The absence of such activity causes imported proteins to incorrectly fold and consequently to aggregate into insoluble complexes (5, 6, 21). Whether hsp60 function is required for the proper metabolism of all mitochondrially targeted proteins whose signal sequence is cleaved by the matrix-processing protease (3) has not been determined, but recent evidence suggests that this may not be so (10). In addition to those studies demonstrating a refolding function for hsp60, models have been proposed (11), based in part on the known functions of the *Escherichia coli* protein groEL (28), a homolog of hsp60 (24), suggesting that hsp60 may function to retain imported molecules in some nonnative form required for the subsequent retransport of such intermediates across the inner mitochondrial membrane. Such a model, the conservative sorting hypothesis (11), is based on strong evolutionary considerations. Recently published evidence (14, 17) supports this model. In addition, it has been suggested (22) that hsp60 participates directly in macromolecular assembly processes, but there is no direct experimental evidence that this is so.

As one way of defining more precisely what step(s) hsp60 may carry out in the refolding/stabilization-retransport/assembly pathway, we developed yeast strains which could be physically depleted of hsp60 or could be functionally depleted by heat inactivating a temperature-sensitive form of the active hsp60 complex, thereby permitting us to determine the initial consequences of losing hsp60 function. Using such strains depleted of functional hsp60, we determined the metabolic fate of a number of cytosolically synthesized mitochondrial proteins that are normally targeted to two different mitochondrial compartments. While all of the proteins that we examined that are normally targeted to the matrix exhibited some targeting or processing defects in the absence of hsp60 function, the metabolism of those normally targeted to the intermembrane space appeared to be normal. The implications of these findings are discussed, especially with respect to current models of intermitochondrial targeting pathways.

MATERIALS AND METHODS

Yeast strains. All *S. cerevisiae* strains used in this study were isogenic to either strain αW303 or strain αW303ΔHSP60, which have been described previously (12, 24). Cells were usually grown on YPGal (2% galactose) or YPD10 (10% glucose) medium unless a selective medium was required for plasmid retention. In these cases, semisynthetic selective medium containing either 2% galactose or 10% glucose was used. In situations where cells were to be grown aerobically, YPEG medium was used.

Plasmids constructed and strains generated. To develop a yeast strain with hsp60 production under galactose control, an *Eco*RI site was introduced by site-directed mutagenesis into the cloned genomic 5.3-kb *Eco*RI fragment containing the *HSP60* gene (24) at 8 bases upstream of the normal translational start site and 26 bases downstream of the normal transcriptional start site. The yeast *GAL10* promoter was isolated as a 0.69-kb *Bam*HI-*Eco*RI fragment from

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plasmid pBM150 (15) and ligated to the 3.2-kb fragment generated by *Eco*RI-*Bam*HI digestion of the altered *HSP60* gene. The 3.9-kb *Bam*HI fragment generated (*GAL10/HSP60*) was ligated into the *Bam*HI site of the polylinker of plasmid pFL39 (a pUC19-derived strain developed by F. LaCroute with *CEN4* and *TRP1* yeast sequences and obtained from Patrick Linder). Yeast strain αW303ΔHSP60 (containing the *HSP60* allele *hsp60::HIS3* and plasmid YEpHSP60 [24]) was transformed with the pFL39 plasmid containing the *GAL10/HSP60* construct. Confirmed transformants were subsequently grown under nonselective conditions on galactose medium for several days, and clones that had lost the YEpHSP60 plasmid by virtue of their ability to grow on plates containing 5-fluoro-orotic acid and galactose (2) were then identified. This was directly verified by Southern analysis of DNA isolated from these cells: none contained a 5.3-kb *Eco*RI fragment hybridizing to an *HSP60* probe (data not shown). This strain or transformants of it were used in all experiments involving hsp60 depletion.

A plasmid (pGR401; developed by Graeme Reid) containing the coding region of the *S. cerevisiae* cytochrome b_2 gene flanked by the promoter and terminator sequences of the yeast alcohol dehydrogenase (*ADH*) gene was obtained from Jeff Schatz. This centromere-containing plasmid, possessing a *URA3* selectable marker, was introduced into both normal and hsp60-depletable strains by standard transformation procedures. In such cells, cytochrome b_2 was constitutively expressed regardless of the carbon source on which the cells were grown.

Generation of mutant strains. Altered forms of the *HSP60* gene were generated either by random mutagenesis of the cloned gene (24), using hydroxylamine treatment and subsequent screening to identify strains expressing temperature-sensitive alleles (2), or by site-directed mutagenesis of the cloned gene and subsequent examination of strains expressing those altered alleles to find ones that endow cells with temperature-sensitive phenotypes. Complete details on the generation and characterization of the mutant strains will be presented elsewhere (unpublished data). The three mutant strains used in this study possess the following altered *HSP60* alleles: (i) the alanine at codon 169 is changed to a valine (*hsp60*^{A169V}); (ii) the serine at codon 222 is changed to a tyrosine (*hsp60*^{S222Y}); and (iii) the glycine at codon 432 is changed to an aspartate (*hsp60*^{G432D}). All three strains grow almost as well as wild-type cells at 30°C, but when shifted to 38°C they continue to divide only two more times and cease any further cell growth by about 6 h after the temperature shift.

Protein extraction, electrophoresis, and Western immunoblot analysis. To immunologically analyze total cell protein extracts, the extracts were initially solubilized by adding 1 part washed cell suspension to 1 part 1.4 M NaOH–5% β-mercaptoethanol. After incubation on ice for 10 to 15 min, an equal volume of acetone and a 1/2 volume of 100% (wt/vol) trichloroacetic acid were added, and the mixture was left on ice for an additional 15 min. The precipitated proteins were collected by centrifugation, washed twice in acetone, dried, and then redissolved in standard sodium dodecyl sulfate (SDS)-containing electrophoresis sample buffer (19). To extract proteins in a native state and to determine the in vivo solubility of particular mitochondrial proteins, frozen pellets of washed cells were physically disrupted by vortexing in the presence of glass beads in ice-cold 0.1 M KCl–0.01 M $MgCl_2$–0.01 M Tris-Cl (pH 7.5)–1% phenylmethylsulfonyl fluoride (PMSF). After cells were broken, Triton X-100 was added to 1%. Following an initial clarifying centrifugation at 1,000 × *g* for 5 min, the extracts were centrifuged at 15,000 × *g* for 15 min to generate a soluble fraction (supernatant) and insoluble fraction (pellet). Earlier experiments (6, 18; unpublished results) showed that the state of solubility of mitochondrial proteins liberated from whole cell extracts by lysis with 1% Triton X-100 was indistinguishable from that resulting from Triton lysis of isolated mitochondria. To prepare proteins for electrophoresis from soluble cell extracts, 2 parts of 3× SDS-gel sample buffer was added to 1 part cell extract sample and boiled. The pelleted proteins were directly dissolved in SDS-gel sample buffer. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on 10 or 12% acrylamide gels, and the proteins were transferred to nitrocellulose filters as described previously (19).

Mitochondrial isolation and fractionation. The method used for mitochondrial isolation is based on that of Daum et al. (8) and has been described previously (19). To disrupt mitochondrial membranes, pelleted mitochondria were suspended in ice-cold 0.1 M KCl–0.01 M $MgCl_2$–0.01 M Tris-Cl (pH 7.5)–1% PMSF, a 1/100 volume of 10% Triton X-100 was added, and the suspension was briefly vortexed. This treatment is sufficient to liberate >95% of the matrix-localized hsp60. The solution is then centrifuged at 12,000 × *g* for 15 min to separate the soluble proteins of the matrix and intermembrane space from the membrane-associated proteins. To further fractionate the soluble proteins liberated by this treatment, the supernatant contents were centrifuged on sucrose density gradients as previously described (10).

Immunological procedures. The anti-hsp60 serum was generated against highly purified yeast hsp60. We obtained our initial antisera against yeast cytochrome c_1 from Mary Crivellone. All other antisera directed against yeast mitochondrial proteins were obtained from Jeff Schatz. Procedures for the use of these antisera in immunodecorating nitrocellulose filters have been published elsewhere (19). To immunoprecipitate mitochondrial proteins liberated from matrix extracts of isolated mitochondria with an antiserum directed against either hsp60 or the mitochondrial form of hsp70 (ssc1p [7]; mhsp70 [25]), 25 μl of a protein A-Sepharose slurry was added to 200 μl of a matrix extract generated as described above. After incubation with mild agitation at 4°C for 30 min, the agarose beads were removed and 50 μl of a slurry of fresh protein A-Sepharose pretreated with 5 μl of antiserum was added, and the suspension was further incubated with mild agitation for 1 h. The beads were collected by gentle centrifugation and washed two times, and the adsorbed proteins were released by incubating the beads in SDS electrophoresis sample buffer at 90°C for 3 min. To immunoadsorb proteins from metabolically labeled cells, total protein extracts of these labeled cells were prepared as described above and then redissolved in 0.1 M KCl–0.01 M $MgCl_2$–0.01 M Tris-Cl (pH 7.5)–1% PMSF–0.1% Triton X-100. Further treatment of these extracts to immunoadsorb the labeled proteins of interest was as described above.

RESULTS

Effect of hsp60 depletion on cell growth and viability. A yeast strain was created in which the only functional copy of *HSP60* was under the control of the *GAL10* promoter (see Materials and Methods). Such cells grew in galactose-containing medium (YPGal) as did wild-type cells, but when transferred to glucose-containing medium (10% glucose; YPD10), their growth ceased in about 25 to 30 h (Fig. 1a). By 20 h after transfer to YPD10 (about six cell generations),

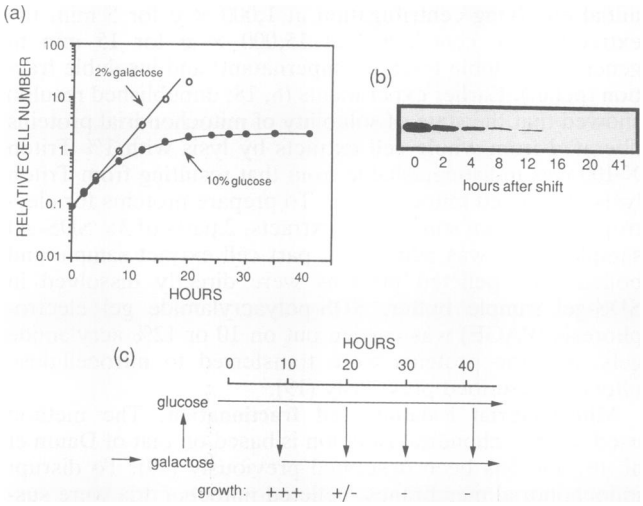

FIG. 1. Growth characteristics of cells expressing hsp60 under the control of the *GAL10* promoter. Cells whose only functional hsp60-coding sequence was under the control of a *GAL10* promoter (see Materials and Methods) were grown in YPGal to mid-log phase at 30°C and then diluted 50-fold into YPGal or YPD10 to an optical density of 600 nm (OD_{600}) of approximately 0.1. Further cell growth at 30°C was monitored spectrophotometrically at 600 nm (a). At intervals, cells in YPD10 were collected by centrifugation, and their total proteins were extracted and then separated by SDS-PAGE. hsp60 was identified in each cell sample by immunoblot analysis using anti-hsp60 antiserum (b). At various times after transfer to YPD10, cells were collected by centrifugation, washed, and resuspended in YPGal, and and further cell growth was monitored spectrophotometrically for an additional 12 h (c). +++, OD_{600} had increased at least 10-fold; +/−, OD_{600} had increased <2-fold; −, there was no increase in OD_{600}. Viable cells counts were also made by plating dilutions of cells on YPGal plates at various times after growth in YPD10. Although some increase in OD_{600} was still observed when transfer to YPGal occurred after 20 h in YPD10, viable cell counts were about 0.1%. After this, no increases in OD_{600} were seen after transfer to YPGal, and viable cell counts were less than 0.01%.

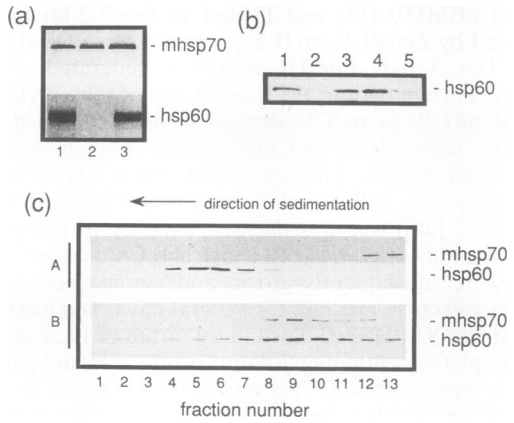

FIG. 2. Characteristics of hsp60 reinduced in cells previously depleted of hsp60. Cells with hsp60 production under galactose control were grown to mid-log phase in YPGal at 30°C and then diluted 50-fold into YPD10 and incubated for an additional 24 h. Cells were collected, washed, resuspended in YPGal, and incubated for an additional 6 h at 30°C. Proteins were isolated from total cell extracts (a) or from mitochondria that had been isolated (b and c) as described in Materials and Methods. (a) Proteins in total cell extracts were separated by SDS-PAGE, transferred to nitrocellulose, and immunodecorated with either anti-hsp60 or anti-mhsp70 serum (a). Lanes: 1, cells grown in YPGal; 2, cells grown in YPGal and transferred to YPD10 for 24 h; 3, cells treated as in lane 2 and then returned to YPGal for 6 h. (b) hsp60 was similarly identified in proteins found in subcellular compartments of fractionated cells treated as in lanes 3 of panel a. Lane 1, initial cell homogenate; lane 2, postmitochondrial supernatant; lane 3, mitochondrial pellet. Mitochondria were resuspended in a solution containing 0.1% Triton X-100, a concentration sufficient to merely disrupt the mitochondrial membranes, and centrifuged to separate the non-membrane-associated material (lane 4) from membrane-associated and insoluble material (lane 5). Approximately equivalent cell amounts were run in each of the five lanes. (c) The 0.1% Triton X-100-soluble material from isolated mitochondria was separated by sucrose density centrifugation as described in Materials and Methods. Fractions were collected, and the proteins in each were analyzed as in panel a. A, proteins from mitochondria of cells grown as in panel a, lane 1; B, proteins from mitochondria of cells grown as in panel a, lane 3.

hsp60 became immunologically nondetectable (Fig. 1b; in control dilution experiments, our lower level of detection was about 1 to 2% of normal levels of hsp60 in wild-type cells grown in YPGal, or about 0.1% of that of cells expressing the *GAL10/HSP60* gene). If returned to YPGal before complete depletion of hsp60, such cells recovered and began growing again. Cells returned to YPGal with as little as 1 to 2% of the amount of hsp60 that they had at the time of the medium shift showed essentially 100% viability. By contrast, cells showing no detectable hsp60 when transferred back to YPGal became morphologically abnormal, and although some small increase in cell number occurred, they eventually died (Fig. 1c). In cells maintained in YPD10 for as long as 35 to 40 h, overall protein synthesis rates, as judged by amino acid incorporation, were depressed by at least 95% relative to that of controls (data not shown).

Cells fully depleted of hsp60, while unable to recover the ability to establish viable colonies under any circumstances, nonetheless initially retained the capacity to reinduce and accumulate nearly normal levels of hsp60 when transferred back to YPGal (Fig. 2a). This newly synthesized protein was processed to its mature form and was found localized in the soluble fraction of detergent-lysed mitochondria (Fig. 2b), presumably the matrix. This reinducibility of pre-hsp60 declined rapidly with time following loss of hsp60, reflecting the loss in overall protein synthetic capacity in these cells, but could still detected as late as 40 h after transfer to YPD10, albeit at a much reduced level (<5%) relative to that seen at 25 or 30 h after the medium shift (data not shown). Furthermore, at these later times, we observed significant accumulation of pre-hsp60 in the cytosol as well as within mitochondria.

Finding newly imported hsp60 in a soluble form was unexpected, as Cheng et al. (5, 6) had shown that import of pre-hsp60 (or other mitochondrially targeted precursors) into mitochondria containing a heat-inactivated, temperature-sensitive form of hsp60 (the product of the *mif4* allele of *HSP60*) resulted in complete insolubilization (in 1% Triton X-100) of the newly imported hsp60 as well as the hsp60 present before the temperature shift. Consequently, as the native form of functional hsp60 is a homodecatetramer (13, 19), we determined the macromolecular form of the newly imported hsp60 in the depleted cells. While the native protein sediments as an approximately 20S particle (19), the newly reinduced hsp60 imported into mitochondria of the previously hsp60-depleted cells was found distributed throughout the gradient, with the majority concentrated near

the top (Fig. 2c). Less than 20% of the hsp60 sedimented in the 20S region of the gradient, and that which did appeared to be part of the overall broad distribution throughout the gradient. A fraction of the material sedimenting in the 20S region of the gradient could conceivably represent a functional hsp60 complex. However, functional hsp60 is essential for viability (24), and the fact that these cells do not recover the ability to divide even when a soluble form of hsp60 is reinduced in cells immediately following depletion of hsp60 we take as evidence that no new functional hsp60 is formed. Additional evidence that hsp60 function is not restored in these cells will be presented later. These data reconfirm the conclusion of Cheng et al. (5) that functional hsp60 is required to assemble new hsp60.

Interaction of newly imported hsp60 with mhsp70 in depleted cells. Recent evidence has shown the essential role that mhsp70 plays in the import of a large number of mitochondrially targeted proteins. In manifesting this role, mhsp70 physically interacts with these imported proteins (16, 25). If the role of hsp60 is to promote refolding (21) and/or assembly (6, 22) of at least some newly imported proteins, then for these imported proteins, an exchange of a physical association with mhsp70 to an association with hsp60 would be required (18). If functional hsp60 were unavailable, release from an mhsp70 association might not occur. As functional hsp60 is required for the refolding and assembly of newly imported hsp60 monomers, we asked whether any of the soluble hsp60 reinduced in depleted cells physically is associated with mhsp70. As a first attempt at finding a potential mhsp70-newly imported hsp60 interaction, we determined the sedimentation properties of mhsp70 in matrix extracts of normal and of functional hsp60-depleted cells (Fig. 2c). While mhsp70 under normal circumstances is found only at the top of the gradient, in depleted cells in which hsp60 has been reinduced, hsp70 is now found in a much broader distribution within the gradient and with a considerable overlap with the newly imported hsp60.

To determine more directly whether a physical association of hsp60 with mhsp70 existed, we examined whether antisera directed against either of these proteins would coprecipitate the other protein. Both of our antisera immunoprecipitated only the protein against which each was directed when tested against matrix extracts of normal cells (Fig. 3). By contrast, immunoprecipitation of detergent-soluble mitochondrial extracts from hsp60-depleted cells reinduced with galactose showed (Fig. 3) that the anti-mhsp70 serum precipitated not only mhsp70 but also hsp60. Similarly, the anti-hsp60 antiserum precipitated hsp60 and coprecipitated mhsp70 as well. The conditions that we used for immunoprecipitation with either antiserum removed more than 90% of the antigen against which each antiserum was directed (data not shown). Thus, the finding that anti-mhsp70 precipitated essentially the same amount of hsp60 as did anti-hsp60 indicates that most, if not all, of the hsp60 in the cell must be in complexes containing at least one mhsp70 molecule. Similarly, while anti-hsp60 also precipitated some mhsp70, the amount was less than that precipitable with anti-mhsp70. Thus, as might be expected, not all mhsp70 was in an association with hsp60. Furthermore, as will be described later, while all of the hsp60 in these cells is soluble following reinduction, some of the mhsp70 becomes insoluble in depleted cells.

Given the heterogeneity and large apparent sizes of the hsp60/mhsp70-containing complexes, it seemed likely to us that other proteins could be trapped in such complexes as well. Immunoprecipitates of depleted cell mitochondrial

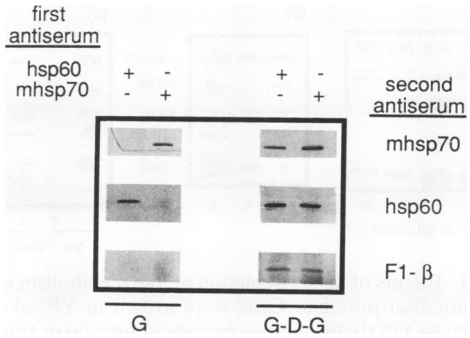

FIG. 3. Immunoprecipitability of hsp60 and mhsp70 in normal and hsp60-depleted cells. Mitochondria were isolated from cells grown in YPGal (G) or those transferred from YPGal to YPD10 for 24 h and then returned to YPGal for an additional 6 h (G-D-G). Triton X-soluble extracts (1% Triton X-100) were prepared as described in Materials and Methods, and anti-hsp60 or anti-mhsp70 antisera were used to adsorb their respective antigens. The proteins adsorbed either directly or indirectly with these antisera were isolated and released, separated by SDS-PAGE, and subsequently identified by immunodecoration of protein blots with an antiserum against mhsp70, hsp60, or F1β.

extracts obtained by using either of the aforementioned antisera showed the mature form of the β subunit of the F1-ATPase (F1β) (1) to be coprecipitated. A similar association was not detected in immunoprecipitates of control cell mitochondrial extracts. This finding suggests that all of these proteins are in common complexes. Presumably, other newly imported proteins would also be found in these complexes. The apparent reasons for these results will be discussed later.

Effect of hsp60 depletion on other mitochondrial-imported proteins. Having examined the metabolism of hsp60 in depleted cells, we then examined the fate of other mitochondrial-targeted proteins in hsp60-free cells. At approximately the time that hsp60 became fully depleted, precursor forms of a number of matrix-targeted proteins began to accumulate (two are shown in Fig. 4a; three others showed similar patterns [data not shown]). In all cases, these precursors copurified with mitochondria and were protease resistant, indicating that they had been imported (data not shown). By contrast, three proteins normally found in the space between the inner and outer membranes either decreased or disappeared (Fig. 4a). As the genes for all three of these proteins exhibit glucose repression to one degree or another, this latter finding was not surprising. However, it did allow us to ask the following: would returning hsp60-depleted cells to galactose medium cause reinduction of these proteins, and if so, in what form(s) would they accumulate? To answer that question, we immunodecorated, with anti-cytochrome b_2 serum, the same protein extracts of depleted and reinduced cells examined earlier (see Fig. 2a). Returning cells to YPGal elicited the synthesis and accumulation of cytochrome b_2 and, more significantly, resulted in the proper processing of this protein to its mature form (Fig. 4b). Similarly, although cytochrome *c* peroxidase (CCPO) had not completely disappeared during the time the cells were in glucose, upon return to YPGal, the level of the completely processed form of this protein increased and again no accumulation of intermediate forms was detected. As the second cleavage step in the processing of pre-cytochrome b_2 and pre-CCPO occurs in the intermembrane space (8, 20, 23, 26), we take this as evidence that the correct targeting of both of these proteins

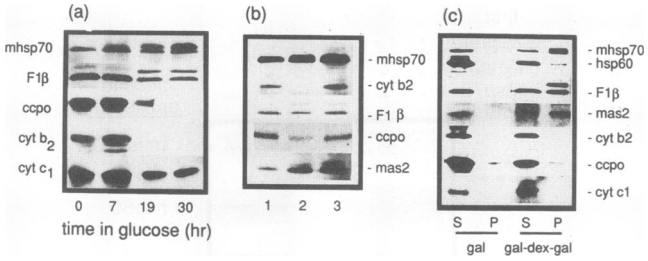

FIG. 4. Effects of hsp60 depletion on the metabolism of a variety of mitochondrial proteins. Cells were grown in YPGal and either transferred to YPD10 for various periods of time (a) or transferred to YPD10 for 24 h and then returned to YPGal for 6 h (b and c). In panels a and b, total cell proteins were isolated and separated by SDS-PAGE, transferred to nitrocellulose, and immunodecorated with an antiserum directed against the mitochondrial protein hsp60, mhsp70, cytochrome b_2 (cyt b_2), F1β, mas2, cytochrome c_1 (cyt c_1), or CCPO. In panel a, we grew cells to early log phase in YPGal (t = 0) and then transferred them to YPD10 for 7, 19, or 30 h. In panel b, the proteins run were from the same cells as those indicated in Fig. 2a. Lanes: 1, cells grown in galactose; 2, cells transferred to glucose for 24 h; 3, cells returned to galactose for 6 h. In panel c, mitochondria were isolated and then solubilized in 1% Triton X-100, and the soluble (S) and insoluble (P) material was separated by centrifugation as described in Materials and Methods. Equal cell equivalents of the proteins extracted from the soluble and insoluble fractions were separated by SDS-PAGE, transferred to nitrocellulose, and immunodecorated with the antisera indicated. dex, dextrose.

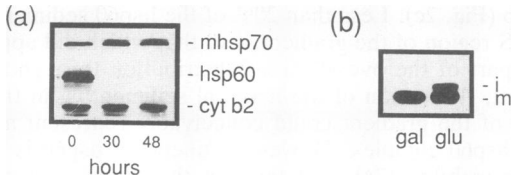

FIG. 5. Cytochrome b_2 synthesis and processing in cells fully depleted of hsp60. Cells containing an *HSP60* gene under the control of the *GAL10* promoter and the cytochrome b_2 gene (*CYB2*) under the control of the *ADH* promoter (see Materials and Methods) were grown to late log phase (OD_{600} = 10) in a galactose-containing semisynthetic selective medium. An aliquot of these cells was diluted 200-fold into a similar medium containing 10% glucose instead of galactose. The increase in cell number was monitored at 600 nm for 48 h, a time by which no further increase in cell number was detectable. Cells were collected at the time of the medium change (t = 0) and again at 30 and 48 h after transfer to the glucose-containing medium. Total protein extracts of collected cells were separated by SDS-PAGE and transferred to nitrocellulose filters. Immunodecoration of these filters (a) was performed with sera directed against hsp60, cytochrome b_2 (cyt b2), and mhsp70. In addition, after 48 h in glucose medium, 8 OD units of cells were collected and concentrated into 1 ml of fresh medium and labeled with 2 mCi of [^{35}S]methionine for 30 min. Total protein extracts were prepared from these cells (and similarly labeled control cells grown in galactose medium), and immunoprecipitation with anti-cytochrome b_2 was carried out as described in Materials and Methods. The precipitated proteins were recovered and separated by SDS-PAGE, and the resultant gel was dried and subjected to fluorography (b). The experimental cells incorporated about 20% as much labeled amino acid as did the controls. Approximately equal amounts of labeled proteins were run in the two lanes. gal, control cells grown in galactose; glu, experimental cells transferred to glucose; m, mature-size cytochrome b_2; i, processed intermediate.

most likely occurred. By contrast, the precursor forms of matrix-localized proteins that had accumulated as a result of hsp60 depletion did not disappear when cells were returned to YPGal, further indicating that hsp60 function had probably not been restored.

As shown by Cheng et al. (5, 6), the loss of hsp60 function by heat inactivation causes a number of newly imported matrix proteins to become insoluble, presumably as a result of their misfolding and aggregation. Therefore, we determined the solubilities of a number of proteins in the depleted and reinduced cells. As seen in Fig. 4c, and as seen before, the majority of the newly imported hsp60 was soluble. However, most of the mature mhsp70 was now insoluble, and all of the precursor form which had accumulated after the initial hsp60 depletion was also insoluble. F1β showed a similar pattern. These results explain why we found only mature forms of these two proteins in our coimmunoprecipitates (Fig. 3). All of the matrix protease subunit (mas2) was in the insoluble fraction in the reinduced cells. By contrast, all three intermembrane space proteins were completely processed to their mature forms and were essentially totally soluble. While these results show the predicted effects of the loss of hsp60 function on matrix-targeted proteins, we cannot rule out the possibility that a small amount of hsp60 function is restored in reinduced cells that is sufficient to allow the proper metabolism of cytochrome b_2, cytochrome c_1, and CCPO but insufficient to permit normal refolding of matrix-localized proteins.

Cytochrome b_2 metabolism in fully depleted cells. To more directly test whether correct cytochrome b_2 processing requires any hsp60 function, we needed to be able to completely deplete cells of hsp60 and at the same time maintain the continued expression of the cytochrome b_2 gene. To accomplish this, we introduced into our *GAL10/HSP60* strain a plasmid containing a copy of the *S. cerevisiae* cytochrome b_2 gene under the control of the *ADH* promoter (pGR401; see Materials and Methods). Wild-type cells transformed with the plasmid containing this construct, when grown on media containing 10% glucose, maintained a steady-state level of immunoreactive cytochrome b_2 greater than that normally seen in nontransformed wild-type cells grown in YPGal (data not shown). To test the effects of hsp60 depletion on cytochrome b_2 metabolism, we grew the cells containing *GAL10/HSP60* and *ADH/CYB2* in galactose-containing, synthetic, selective media (in order to maintain the *ADH/CYB2*-containing plasmid) and then transferred some cells to selective media containing 10% glucose. Because of the reduced growth rates of these cells in selective media, hsp60 depletion required >30 h (by dilution of the zero-time control, we estimate that 30-h cells contain about 2% as much hsp60), but by 48 h, no hsp60 (<0.2% of the zero-time controls) was immunologically detectable (Fig. 5a). The cellular concentrations of cytochrome b_2 remained essentially unchanged throughout the course of the experiment, and no precursor or intermediate forms were detected even at 48 h. By contrast, as we had seen before, as hsp60 disappeared, the precursor from of mhsp70 began to accumulate even by 30 h and had increased at 48 h. As the total cell number actually doubled between 30 and 48 h and the measured protein content more than doubled, at least one-half of the protein seen in 48-h cells had to have been accumulated between 30 and 48 h. As hsp60 is barely present at 30 h and is nondetectable at 48 h, this finding clearly suggests that hsp60 function is not essential for continued normal cytochrome b_2 metabolism. To show this more directly, cells that had been in 10% glucose medium for 48 h

were labeled with [^{35}S]methionine for 20 min. We then immunoprecipitated protein extracts of these cells with anti-cytochrome b_2 serum and compared the labeled immunoprecipitable proteins with those obtained by the same protocol from control cells grown in galactose (Fig. 5b). While the overall rate of protein synthesis in the glucose-grown cells was only about 20% of that of controls, and some nonmature form of cytochrome b_2 was detected, these cells accumulated primarily a fully processed form of cytochrome b_2. We attempted to prepare mitoplasts from these cells in order to unequivocally identify the location of the cytochrome b_2. However, because of the physiological state of these cells, we were unable to prepare mitoplasts and ascertain the exact location of the newly synthesized cytochrome b_2. Thus, it remains formally possible that in these admittedly compromised cells, the second cleavage artifactually took place in the matrix.

Effects of heat inactivation of hsp60 on the metabolism of intermembrane space proteins. In our results with hsp60-depleted cells, we consistently found adverse effects of the loss of hsp60 function on matrix-targeted proteins, while under the same experimental conditions, we saw essentially no effects on proteins destined for the intermembrane space. However, the time required for complete hsp60 depletion in these experiments was such that both biochemically and physiologically, cells were extremely abnormal. Another way of abolishing hsp60 function more rapidly is to selectively inactivate the hsp60 complex (6). Having developed a number of new mutant alleles of *HSP60* which confer a conditional lethal phenotype (temperature sensitivity) on cells expressing them (unpublished data), we chose three strains in which hsp60 function appeared to be inactivated quite rapidly (in less than 2 h), but unlike in the strain expressing the *mif4* allele of *HSP60* (6), the hsp60 complex does not rapidly precipitate at heat-inactivating temperatures (Fig. 6). With such strains, we should be able to initially abolish hsp60 function while generating a minimum of secondary pleiotropic effects. Using such cells, we examined the metabolism of three newly synthesized matrix proteins at permissive (30°C) and nonpermissive (38°C for 3 h) temperatures (Fig. 7a to c). In all cases, at 38°C in mutant cells, the predominant forms of all immunoprecipitable, pulse-labeled proteins appeared in the insoluble fraction of mitochondrial extracts. The absence of detectable precursors of all three proteins either inside mitochondria or in the cytosol (data not shown) indicated that the heat inactivation of hsp60 had no detectable side effects on import and processing. While the two strains expressing the *HSP60* alleles *hsp60*^{G432D} and *hsp60*^{A169V} showed some newly synthesized matrix-targeted proteins remaining soluble at the nonpermissive temperature, the strain expressing *hsp60*^{S222Y} produced essentially no soluble proteins at all. In all strains, however, hsp60 inactivation had a clearly negative effect of the same relative magnitude on the production of soluble forms of each of the three matrix proteins.

As all three mutant strains were respiratorily competent, we could grow cells on nonfermentable carbon sources and thereby heat inactivate hsp60 without adversely affecting the synthesis of those proteins that are glucose repressible, i.e., the intermembrane-targeted proteins. These proteins, cytochrome b_2 and cytochrome c_1, were synthesized, processed, and remained totally soluble (Fig. 7d and e) in all three strains under the same nonpermissive conditions which negatively affected the metabolism of the three matrix-targeted proteins. Given the similarity of results with these three strains, we conclude that inactivation of hsp60 function

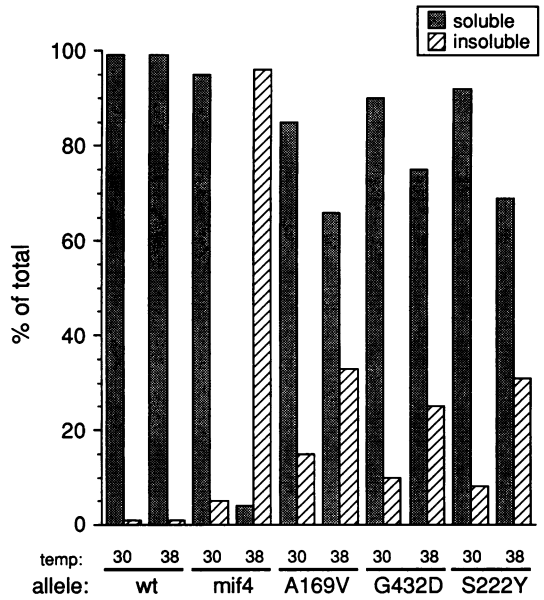

FIG. 6. Effects of elevated temperature on hsp60 solubility in wild-type (wt) and mutant cells. Cultures of cells growing in YPEG at 30°C were divided in half. One half remained at 30°C; the other was transferred to 38°C for 4 h. Cells were then collected and disrupted with glass beads, and 1% Triton X-100-soluble and -insoluble material was separated by centrifugation. Equivalent cellular amounts of the soluble and insoluble protein fractions were separated by SDS-PAGE, and the amount of hsp60 in each was determined immunologically. The resulting stained Western blots were scanned, and the relative amount of the total cellular hsp60 represented in the soluble and insoluble fractions was calculated.

(most dramatically seen in the strain expressing *hsp60*^{S222Y}) has no effect on the proper metabolism of cytochrome b_2 and cytochrome c_1. Because of the apparently normal targeting, import, and processing of all of the mitochondrial proteins that we examined, it seemed entirely unlikely to us that the second processing step of cytochrome b_2 and cytochrome c_1 in these mutant strains could have occurred anywhere but at the outer surface of the inner membrane. Nevertheless, we did determine that the cytochrome b_2 synthesized at 38°C in the strain expressing *hsp60*^{S222Y} had the same protease sensitivity in intact mitochondria (resistant) and in mitoplasts (sensitive) as that shown by the pre-existent cytochrome b_2, indicating to us that both were in the intermembrane space (data not shown). As our results with the mutant strains are completely consistent with the earlier depletion results, we conclude that hsp60 function is not required for the correct targeting of at least the two intermembrane proteins cytochrome c_1 and cytochrome b_2.

DISCUSSION

In a number of different experiments, we abolished hsp60 function within mitochondria, thereby causing newly imported matrix-targeted proteins to become insoluble (presumably because they are not correctly folded following import) and, in some cases, not fully processed. Under these same experimental conditions, the intermembrane-targeted proteins cytochrome b_2, cytochrome c_1, and CCPO were correctly processed to their mature forms and were found as fully soluble (presumably correctly folded) proteins. Whether this latter finding is due to the absence of an

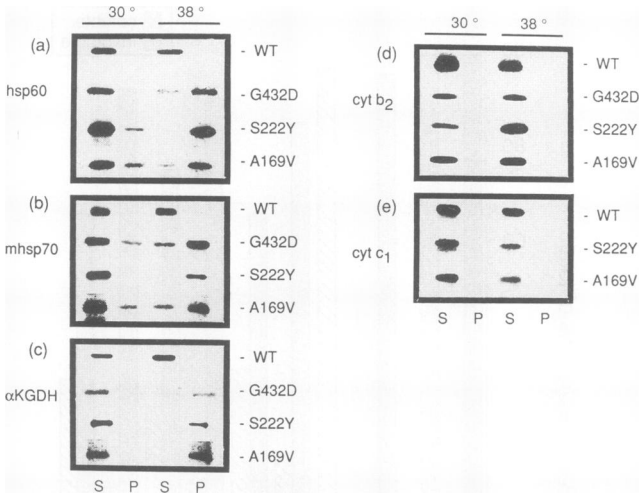

FIG. 7. Solubilities of newly imported mitochondrial proteins in wild-type (WT) and three temperature-sensitive strains. Cultures of cells growing in YPEG at 30°C at an OD_{600} of 0.5 to 1.0 were divided in half. One remained at 30°C; the other was shifted to 38°C for 3 h. Then [^{35}S]methionine was added to each culture at 150 μCi/ml, and the cells were incubated for an additional hour. At the end of the labeling period, the cultures were chilled on ice, the cells were collected and washed by centrifugation, and the cell pellets were frozen. Protein extracts of the Triton X-100-soluble (S) and -insoluble (P) fractions from cells labeled at the two temperatures were prepared as described in Materials and Methods (see reference 4 for further details). Sequential immunoprecipitations of each of the labeled extracts with antisera directed against hsp60 (a), mhsp70 (b), α-ketoglutarate dehydrogenase (αKGDH) (c), cytochrome b_2; (cyt b_2; d), and cytochrome c_1 (cyt c_1; e) were carried out as described in Materials and Methods and reference 4. Equivalent cellular amounts of the immunoadsorbed proteins from the soluble and insoluble extracts were separated by SDS-PAGE, and the resulting gels were subjected to fluorography. Each of the three mutant strains examined, expressing a temperature-sensitive allele of *HSP60* (G432D, S222Y, or A169V), had a single nucleotide base change in its *HSP60* coding region, causing the production of a conditionally functional hsp60.

absolute requirement for hsp60 interaction with these three proteins, assuming that they pass through the matrix as predicted by the conservative sorting model, or whether these proteins never fully enter the matrix space (only their signal sequences need enter for the first processing step to occur), thereby never physically encountering hsp60, cannot be determined from our experiments. We cannot rule out the possibility that when present, hsp60 interacts with and stabilizes the intermediate forms of each of these proteins (17). On the other hand, our results are completely consistent with the recent demonstration (10) that in vitro import, processing, and targeting of two intermembrane space proteins can occur in mitochondria fully depleted of ATP. In these experiments, the intermediate forms of neither of these proteins were detectable in the matrix but were found only in the intermembrane space. Having no functional hsp60 in the mitochondrion should not interfere with a transport pathway which does not require passage through the matrix. Thus, our data do not support the suggestion that hsp60 plays a necessary role in the facilitation of a conservative sorting pathway (6, 17) at least for cytochrome b_2, CCPO, and cytochrome c_1. Our data are consistent with the stop-transfer model (8, 9) of mitochondrial targeting.

One apparently anomalous result that we present is the fact that at least some processing of mitochondrial-targeted precursors continues in hsp60-depleted mitochondria and at the same time we find that the mas2 protein, one of the two subunits of the matrix protease, becomes essentially totally insoluble. Interestingly, and of direct relevance, when hsp60 is heat inactivated in the yeast strain α143, which contains a temperature-sensitive allele (*mif4*) of *HSP60* (6), the soluble matrix protease present in the mitochondria of these cells prior to the heat shift becomes insoluble shortly after the temperature increase (6, 10). In this case, precursor processing also continues in these cells (6). The simplest (although not necessarily correct) interpretation of these results is that "insolubility" is an isolation artifact. That is, the matrix protease is not really insoluble in the mitochondrial matrix, but upon detergent lysis of mitochondria, it aggregates for some unknown reason and becomes sedimentable at relatively low *g* forces. Hence, it becomes insoluble by our operational definition. In any event, processing protease activity is retained in mitochondria from which, by detergent lysis, the proteins responsible for this activity are not insoluble in their normally soluble, dimeric form (27).

While we found that the reinduction of synthesis of hsp60 in depleted cells resulted in the accumulation of a processed and soluble product, Cheng et al. (5) found that induction of de novo hsp60 synthesis in heat-treated α143 cells resulted in the accumulation of a processed but insoluble product. We assume, but cannot prove, that the retention of solubility of hsp60 in our system is in some way due to the continued interaction of newly imported hsp60 with mhsp70. If this interpretation is correct, why would not the mhsp70 in α143 mitochondria play the same role in maintaining the imported hsp60 in a soluble form, assuming of course that the mhsp70 itself remains soluble? While the two experimental protocols are similar in many respects, one significant difference was that while we reinduced hsp60 in depleted cells at 30°C, Cheng et al. induced new hsp60 synthesis in α143 cells at 37°C. One explanation for the differences that we report might be that the hsp60-mhsp70 complex is formable and stable at 30°C but not at 37°C. Destruction of this association or prevention of it in the first place could then lead to aggregation and precipitation of hsp60. As a test of this idea, we examined the solubility of newly synthesized hsp60 in depleted cells (25 h in YPD10) returned to galactose and simultaneously shifted to 37°C for 6 h. The hsp60 reinduced at 37°C showed essentially the same solubility as did that reinduced at 30°C (data not shown). Thus, the temperature difference is not the reason for the alternative results. The reason for this discrepancy remains to be determined.

While our data clearly indicate a physical association between newly imported hsp60 and mhsp70 in mitochondria depleted of functional hsp60, the stoichiometry of that association is uncertain (Fig. 2c). Clearly, it is not one to one, and there is a broad distribution of different-size aggregates containing both of these two proteins. Because all hsp60 can be immunoprecipitated with anti-mhsp70 antibody but the converse is not true when anti-hsp60 antibody is used, we cannot determine the relative distributions of the two proteins in these complexes. What is more, F1β must be associated with one or both of these proteins, as it can be coprecipitated with either antiserum. However, we have not determined the distribution of F1β in these complexes. Moreover, despite the fact that we did not immunologically identify other proteins coprecipitable with anti-mhsp70, still other proteins may well be included in these complexes. By more direct biochemical analysis of mitochondrial extracts

of depleted cells, it should be possible to define more precisely the nature of these interactions.

Finally, it is not clear why we even find a stable complex containing hsp60 and mhsp70 in our cells. Manning-Krieg et al. (18) found that newly imported hsp60 monomers, still in a protease-sensitive form, are released from their association with mhsp70 prior to becoming associated with a functional hsp60 complex. Thus, on the basis of these results, one would have predicted that released monomers, unable to be correctly refolded by a functional hsp60 complex in depleted cells, would have simply aggregated and become insoluble. However, as we saw a change in the solubility properties of mhsp70 upon depletion, it may be that mhsp70 function is also compromised, thereby accounting for the nonrelease of the imported hsp60 which had become bound to mhsp70. The answer to this anomaly remains to be determined.

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