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Cyclophilin 20 Is Involved in Mitochondrial Protein Folding in Cooperation with Molecular Chaperones Hsp70 and Hsp60

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We studied the role of mitochondrial cyclophilin 20 (CyP20), a peptidyl-prolyl *cis-trans* isomerase, in preprotein translocation across the mitochondrial membranes and protein folding inside the organelle. The inhibitory drug cyclosporin A did not impair membrane translocation of preproteins, but it delayed the folding of an imported protein in wild-type mitochondria. Similarly, *Neurospora crassa* mitochondria lacking CyP20 efficiently imported preproteins into the matrix, but folding of an imported protein was significantly delayed, indicating that CyP20 is involved in protein folding in the matrix. The slow folding in the mutant mitochondria was not inhibited by cyclosporin A. Folding intermediates of precursor molecules reversibly accumulated at the molecular chaperones Hsp70 and Hsp60 in the matrix. We conclude that CyP20 is a component of the mitochondrial protein folding machinery and that it cooperates with Hsp70 and Hsp60. It is speculated that peptidyl-prolyl *cis-trans* isomerases in other cellular compartments may similarly promote protein folding in cooperation with chaperone proteins.

Mitochondria import most of their proteins from the cytosol. The import pathway of mitochondrial proteins encoded by the nucleus can be divided into three main steps: (i) targeting of the preproteins to mitochondria, (ii) translocation across the mitochondrial outer and inner membranes, and (iii) maturation and folding of the proteins (summarized in references 3, 40, and 59). The mitochondrial outer membrane carries receptor proteins and a general insertion pore that mediate the recognition and membrane insertion of preproteins. The protein import machinery of the mitochondrial inner membrane, consisting of the integral membrane proteins Mim17 (Sms1p) and Mim23 (Mas6p) and the peripheral membrane protein Mim44 (Isp45), was recently identified (14, 34, 40, 50). The heat shock protein Hsp70 in the matrix plays a central role in the import of preproteins. It associates with Mim44 in a nucleotide-sensitive and reversible manner (29, 40, 45, 53). Mitochondrial Hsp70 (mt-Hsp70) directly binds the polypeptide chain in transit and drives its translocation into the matrix. Furthermore, mt-Hsp70 is an initial component of the refolding machinery in the matrix and promotes transfer of the preprotein to the "chaperonin" system Hsp60/Cpn10 (33, 39, 46, 49, 59). Recently two partner proteins of mt-Hsp70 were identified, the mitochondrial homologs Mdj1p and Mge1p of the bacterial heat shock proteins DnaJ and GrpE (4, 10, 25, 31, 37, 48, 68). Mdj1p and Mge1p are assumed to be involved in the folding pathway of preproteins and to promote the transfer to Hsp60. In addition, Mge1p participates in the membrane translocation of preproteins (68).

We asked if additional partner proteins are involved in membrane translocation or folding of mitochondrial proteins. Several years ago, a 20-kDa cyclophilin (CyP20) in the matrix of *Neurospora crassa* mitochondria was identified (65). Mitochondrial CyP20, the function of which was unknown to date, belongs to the growing family of cyclophilins that are characterized by binding of the immunosuppressive drug cyclosporin A (CsA) (19). Known functions of members of the cyclophilin family are as follows. (i) In higher eukaryotes, complexes between CsA and cytosolic isoforms of cyclophilin mediate immunosuppressive effects by interference with calcineurin-dependent signal transduction pathways (30, 55). Recently a human cyclophilin was found to be involved in calcium signalling in T cells (6). *N. crassa* and yeast strains lacking cyclophilin are resistant to CsA (7, 64). (ii) Other cyclophilins are components of steroid hormone receptors (42). (iii) In vitro studies with purified cyclophilins showed that they form a family of peptidyl-prolyl *cis-trans* isomerases (PPIases). PPIases catalyze the *cis-trans* isomerization of Xaa-Pro bonds of some proteins and thereby accelerate protein folding in vitro (15, 52). (iv) Several observations suggest a role for cyclophilins, e.g., for the *NinaA* gene product in *Drosophila melanogaster*, in protein maturation processes in the secretory pathway, in particular in the endoplasmic reticulum (2, 9, 38, 54, 57). Addition of CsA was shown to interfere with the maturation of some proteins in the endoplasmic reticulum (23, 32, 58). (v) In *Saccharomyces cerevisiae*, the expression of a cytosolic cyclophilin and of an endoplasmic reticulum cyclophilin is increased by heat shock (61). Deletion of either cyclophilin as well as deletion of a cyclophilin with a presumptive mitochondrial location increases the sensitivity of yeast cells to heat stress (11, 61). Points iii to v above are suggestive of an involvement of cyclophilins in cellular protein folding processes.

Here we studied the role of CyP20 in the mitochondrial matrix in the import and folding of proteins. Isolated mitochondria turned out to form a very suitable system to study cellular protein transport and folding reactions. In particular, reactions occurring in the matrix of isolated mitochondria closely resemble the in vivo situation, and therefore a detailed analysis of the functions of molecular chaperones in transport and folding of proteins was possible (16, 26, 35, 39). We report that mitochondria lacking CyP20 are not impaired in membrane translocation of preproteins. However, folding of an imported protein is delayed in cyclophilin-deficient mitochon-

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dria. CyP20 seems to be involved in protein folding in cooperation with the heat shock proteins Hsp70 and Hsp60.

MATERIALS AND METHODS

Import and folding of preproteins in mitochondria. Protocols were designed according to published procedures for isolating mitochondria from wild-type *N. crassa* (strain 74A) and the *csr-1* mutant (allele B12) (64); import of mitochondrial preproteins from reticulocyte lysate and from 8 M urea; dissipation of the membrane potential with valinomycin, antimycin A, and oligomycin; and treatment of mitochondria with trypsin or proteinase K (39, 41). For import from urea, the ^{35}S-labeled precursor protein pSu9-dihydrofolate reductase (DHFR) was precipitated from 25 μl of reticulocyte lysate with 66% ammonium sulfate at 0°C. The pellet was dissolved in 20 μl of a solution containing 8 M urea, 2 mM dithiothreitol, and 50 mM MOPS (morpholinepropanesulfonic acid)-KOH (pH 7.2) and shaken for 60 min at room temperature. Isolated mitochondria (50 μg of protein) were preincubated at 25°C in 100 μl of bovine serum albumin (BSA) buffer (3% BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM NADH, 250 μM ATP, 10 mM MOPS [pH 7.2]) for 3 min. The precursor was added in 2.5 μl of urea solution, and the sample was mixed immediately. The reaction was stopped by dilution of the import assay into 300 μl of ice-cold SEM (250 mM sucrose, 1 mM EDTA, 10 mM MOPS [pH 7.2]) containing 5 mM ADP. The mitochondria were reisolated by centrifugation and resuspended in 100 μl of SEM. Residual precursor proteins outside the mitochondria were removed by treatment with trypsin (15 μg/ml) for 5 min. The reaction was stopped by the addition of soybean trypsin inhibitor (900 μg/ml). Wild-type and *csr-1* mitochondria were fully intact under the experimental conditions for folding reaction times of up to 20 min. The limited stability of isolated mitochondria in the in vitro import system precluded a detailed analysis at longer incubation times. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography and subjected to quantitation by two-dimensional (2D) densitometry.

Inhibition of protein folding by CsA. Prior to contact with CsA, all tips and tubes were rinsed with ethanol. Stock solutions of CsA were prepared in ethanol and diluted 1:50 into aqueous solutions with rapid mixing. Samples of different CsA concentrations were adjusted to the same ethanol concentration. Mitochondria were prewarmed to 25°C and then incubated with CsA for 10 min. The mitochondria were then reisolated, and CsA was added to the import assays at the same concentrations as in the preincubation. Refolding experiments in vitro were performed on ice with 40-μl samples containing 75 mM KCl, 3 to 7.5 μM CyP20, 20 μg of cytochrome *c* (to facilitate quantitative trichloroacetic acid [TCA] precipitation, the same results were obtained with soybean trypsin inhibitor), and 60 mM MOPS-KOH (pH 7.2). CsA was added in a 20-fold molar excess over CyP20 (in a manner similar to that of other in vitro refolding assays [15, 67]). The preprotein was prepared in the way described for the import reactions and diluted 40-fold. Folding reactions were stopped by the addition of proteinase K at a concentration of 200 μg/ml. After 10 min of incubation, the samples were treated with phenylmethylsulfonyl fluoride (PMSF) and subjected to precipitation with TCA.

Immunoprecipitation of proteins after import. For coimmunoprecipitations of imported proteins, mitochondria (50 μg of protein) were lysed in 200 μl of Triton X-100 buffer containing 1% Triton X-100, 100 mM NaCl, 5 mM EDTA, and 10 mM Tris-HCl (pH 7.2). After a clarifying spin of 10 min at 20,000 × *g*, the supernatant was added to antibodies prebound to protein A-Sepharose. An additional 300 μl of Triton X-100 buffer was added, and the samples were incubated for 45 min at 4°C with gentle shaking. The protease sensitivity of Hsp70-bound protein was assayed by treatment with 25 μg of proteinase K per ml at 0°C.

Isolation of CyP20 and sequencing of the CyP20 gene. CyP20 was isolated from *N. crassa* as described previously (65). Genomic DNA was isolated from the wild-type strain and from the *csr-1* mutant by the method of Borges et al. (5). The genes were amplified by PCR using a mixture of *Taq* and *Pwo* DNA polymerase (Expand Long Template PCR System; Boehringer) and combinations of primers 1 (GCGCTTGACCCAACACCCTG [positions 32 to 51]) and 3 (GTTTACGT GATCTCATCAAC [positions 2561 to 2580]) and primers 2 (CATTGCCGT CAGCGTCAGGG [positions 922 to 941]) and 3, respectively. Primers were designed according to the published wild-type sequence (63). The amplified DNA fragments were cloned in pGEM4 (Promega) and sequenced by using Sequenase version 2.0 (Amersham) and gene-specific primers. Five independent clones each from the wild-type strain and the *csr-1* mutant were sequenced on both strands.

RESULTS

Effect of CsA on import and folding of pSu9-DHFR in mitochondria. With the precursor protein Su9-DHFR, a reliable assay which is widely used to monitor the folding of minute amounts of protein upon import into the matrix of isolated mitochondria has been developed (26, 35, 39, 48). pSu9-DHFR was constructed as a fusion protein between the presequence of F_0-ATPase subunit 9 and the entire mouse DHFR (41). After translocation into the mitochondrial matrix the preprotein is cleaved to mature-sized DHFR (plus 3 amino acids of mature Su9). DHFR is a monomeric single-domain protein with a molecular mass of 21.5 kDa that acquires a high protease resistance only in its correctly folded conformation.

To analyze possible functions of cyclophilins in the import of mitochondrial preproteins, we tested whether the specific inhibitor CsA affects the translocation of pSu9-DHFR across the mitochondrial membranes. The preprotein was synthesized in reticulocyte lysate in the presence of [^{35}S]methionine, precipitated with ammonium sulfate, and dissolved in 8 M urea. After 1 h of shaking at room temperature to allow equilibration in the *cis-trans* isomerization of prolyl bonds, the urea solution was diluted into import assays with isolated mitochondria. As shown in Fig. 1A, the protein was efficiently imported into mitochondria. Its presequence was cleaved, and the protein became resistant against externally added proteases. CsA did not interfere with translocation (Fig. 1A), suggesting that CsA-binding proteins are not involved in rate-limiting steps of membrane translocation of pSu9-DHFR.

Does CsA interfere with the fate of the imported preprotein after translocation? For the experiment whose results are shown in Fig. 1B, unfolded pSu9-DHFR was imported into isolated mitochondria, and import was stopped by dissipation of the membrane potential ($\Delta\psi$) after 30 s. After different times for refolding of the imported DHFR domains at 25°C, the mitochondria were rapidly cooled to 0°C, treated with protease to remove nonimported preproteins, and reisolated. After lysis with detergent (in the presence of EDTA and without addition of ATP), the folding state of the imported DHFR molecules was determined by treatment with proteinase K (26, 39). By this assay we found that treatment of mitochondria with CsA delayed folding of the imported protein (Fig. 1B). This result indicated that CsA inhibited a component which is involved in the refolding reaction. A candidate for this component is CyP20, a 20-kDa cyclophilin which is a soluble protein in the mitochondrial matrix of *N. crassa* (65).

CyP20 accelerates folding of Su9-DHFR in vitro. We isolated CyP20 from *N. crassa* and tested whether the refolding of denatured pSu9-DHFR is affected by CyP20 in vitro. The ^{35}S-labeled preprotein was unfolded in 8 M urea and then diluted into buffer at 0°C. At different time points the samples were treated with protease to determine the amount of folded protein (Fig. 2). While the presequence of pSu9-DHFR remained protease sensitive under all conditions, the DHFR domain became resistant (Fig. 2A). The initial rate of refolding was accelerated three- to fourfold by CyP20 (Fig. 2B), suggesting that CyP20 acts as a prolyl *cis-trans* isomerase in the refolding reaction. The effect of CyP20 was inhibited by CsA (Fig. 2A; compare lane 5 with lane 6). We conclude that Su9-DHFR is a substrate for CyP20 in vitro.

An *N. crassa* mutant with a defective CyP20 gene. In the following experiments we made use of the CsA-resistant *N. crassa* strain B222-5 (allele B12), which lacks immunodetectable cytosolic and mitochondrial CyP20 (64). This strain carries the mutation *csr-1* in a wild-type background. The mutation has been located distal from *his-2* on the right arm of chromosome I (64). We confirmed that the *csr-1* mutation is located within the CyP20 gene that encodes both the cytosolic and mitochondrial isoforms. Genomic DNA was isolated from the mutant strain and from the corresponding wild-type strain. The CyP20 gene of the *csr-1* mutant was completely sequenced. Two base pair changes were detected, a deletion of nucleotide T2249 and a change of nucleotide T2251 to a C in

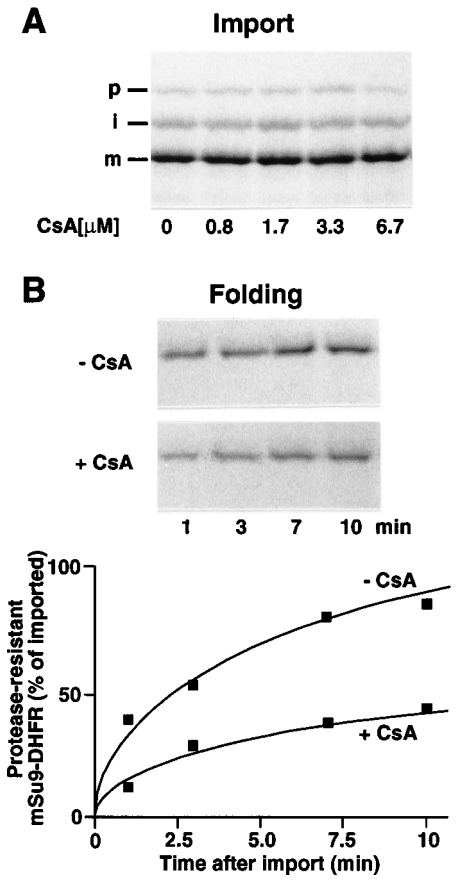

FIG. 1. Import and folding of pSu9-DHFR in wild-type mitochondria in the presence of CsA. (A) Import of pSu9-DHFR in the presence of CsA. Isolated mitochondria from *N. crassa* were preincubated with different concentrations of CsA. The ^{35}S-labeled preprotein was synthesized in reticulocyte lysate and imported by dilution from 8 M urea. After incubation for 6 min at 25°C, the mitochondria were reisolated and treated with trypsin. The imported proteins were analyzed by SDS-PAGE and fluorography. (B) Effect of CsA on refolding of imported pSu9-DHFR. Mitochondria were preincubated in the presence or absence of 5 μM CsA. The import reaction of pSu9-DHFR was stopped after 30 s by the addition of valinomycin, antimycin A, and oligomycin. After further incubation at 25°C for the times indicated (to allow refolding of the imported protein), samples were rapidly cooled on ice, reisolated, treated with trypsin, and then lysed by the addition of detergents. The samples were mixed with proteinase K (100 μg/ml) and incubated at 0°C for 10 min. After the addition of PMSF the samples were subjected to TCA precipitation and analyzed by SDS-PAGE, fluorography, and densitometry. Shown are the percents proteinase K-resistant protein relative to the total amounts of imported protein. p, i, and m, precursor-, intermediate-, and mature-sized forms of Su9-DHFR, respectively.

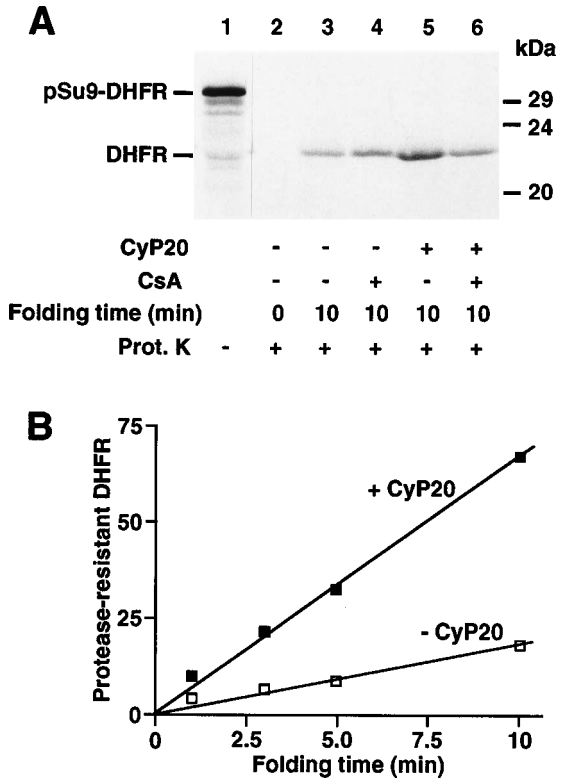

FIG. 2. Folding of Su9-DHFR in vitro. (A) Effects of CyP20 and CsA on refolding of pSu9-DHFR. ^{35}S-labeled pSu9-DHFR was translated in reticulocyte lysate, precipitated with ammonium sulfate, and dissolved in a solution containing 8 M urea, 2 mM dithiothreitol, and 50 mM MOPS-KOH (pH 7.2). The solution was diluted 50-fold into 75 mM KCl–60 mM MOPS-KOH (pH 7.2) at 0°C, containing CyP20 and CsA as indicated (and as described in Materials and Methods). The sample in lane 2 additionally contained 200 μg of proteinase K (Prot. K) per ml; to the samples in lanes 3 to 6 the protease was added after an incubation of 10 min. The proteinase K reaction was stopped by the addition of PMSF, and the proteins were precipitated with TCA and analyzed by SDS-PAGE and fluorography. (B) Refolding of pSu9-DHFR in the presence and in the absence of CyP20. After dilution of pSu9-DHFR from 8 M urea, samples were treated with proteinase K at the indicated times. After SDS-PAGE and fluorography, the relative amount of refolded protein was determined by densitometry and corrected for the different numbers of methionines in pSu9-DHFR and DHFR.

the fourth exon of the CyP20 gene (Fig. 3A; numbering is according to that of reference 63). As a consequence, the *csr-1* cyclophilin (which in the wild-type strain has a length of 179 amino acids) was altered, starting from amino acid residue 139 and continuing into an unrelated sequence of 10 amino acids. By Northern (RNA) blotting we confirmed that transcription of the gene was not reduced and that the two CyP20 mRNAs were retained at virtually the same length as in the wild-type strain (Fig. 3B) (the less abundant larger mRNA codes for the mitochondrial isoform, which is synthesized with a presequence [65]). In Western blots (immunoblots) of mutant mitochondria, no CyP20 or related smaller proteins were detectable by polyclonal antibodies raised against the authentic cyclophilin (Fig. 3C). Since CyP20 was also absent in cytosolic fractions (64), the shortened mutant proteins appear to be unstable and are most likely rapidly degraded after synthesis. This assumption was supported by translation of the mutant mRNAs in rabbit reticulocyte lysates in vitro (data not shown).

Mitochondria lacking CyP20 efficiently import preproteins. Preproteins were ^{35}S labeled during synthesis in rabbit reticulocyte lysates and incubated with energized isolated mitochondria. Import was assessed by monitoring the removal of the presequences and the protection of the imported proteins against externally added protease (56). We used the precursors of the inner membrane proteins cytochrome c_1 and ADP/ATP carrier and the matrix-targeted proteins F_1-ATPase subunit β and pSu9-DHFR (41). The preproteins were imported into *csr-1* mitochondria at a rate close to that for import into wild-type mitochondria, independently of whether the precursors were directly imported from reticulocyte lysate (Fig. 4A through C) or were unfolded in 8 M urea prior to import (Fig. 4D). These results are in agreement with the lack of inhibition by CsA, and we conclude that translocation of preproteins into mitochondria does not require CyP20 in the matrix. Since protein translocation strictly depends on a $\Delta\psi$ and functional matrix Hsp70 (16, 26, 56), and processing requires the function of both subunits of the matrix processing peptidase (1), the

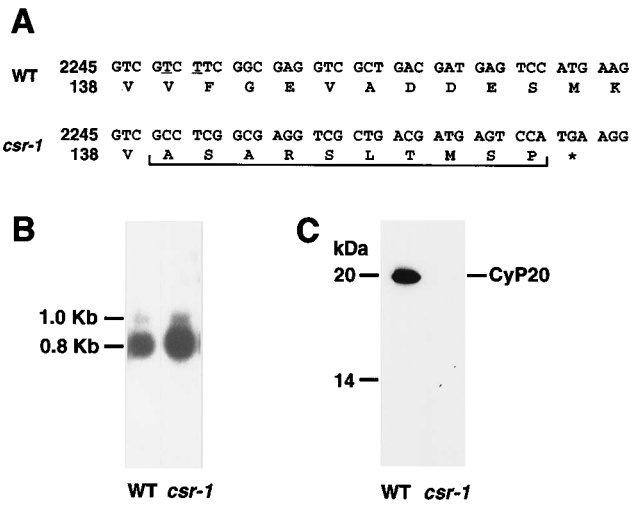

FIG. 3. The *N. crassa* strain *csr-1* contains a mutation in the coding sequence of the CyP20 gene. (A) DNA sequence and deduced amino acid sequence of CyP20. The numbering of nucleotides is according to the published sequence (63), and the numbers of the amino acid residues indicate the position within the mature CyP20 protein. Nucleotides which are mutated in the *csr-1* mutant are underlined in the wild-type (WT) sequence. Amino acid residues 139 to 148 of the mutant (underlined) are altered because of a frameshift, and the asterisk marks a stop codon. (B) Northern blot. Poly(A)$^+$ RNA preparations from *N. crassa* were subjected to electrophoresis on a denaturing agarose gel containing formaldehyde. The nucleic acids were blotted onto a nylon filter and probed with a nick-translated CyP20 cDNA. The upper band (1.0 kb) corresponds to the mRNA coding for mitochondrial CyP20, and the lower band (0.8 kb) corresponds to the mRNA coding for cytosolic cyclophilin (65). (C) Western blot. Mitochondrial proteins (80 μg of protein per lane) were resolved by SDS-PAGE and transferred to nitrocellulose, and CyP20 was visualized with a polyclonal antiserum raised against isolated CyP20.

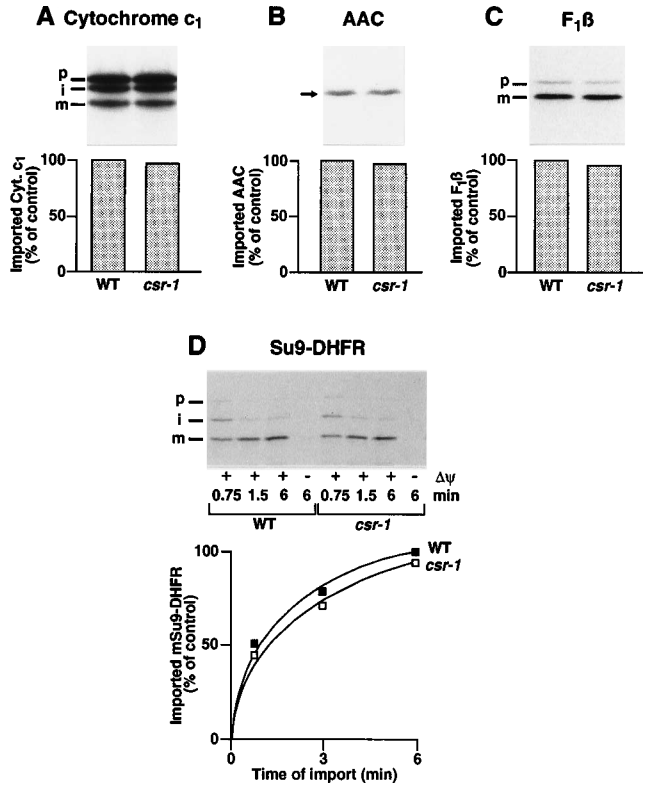

FIG. 4. Cyclophilin-deficient mitochondria import precursor proteins. (A to C) Import of the precursors of cytochrome c_1 (Cyt c_1), ADP/ATP carrier (AAC; arrow), and F_1-ATPase subunit β (F_1β) from reticulocyte lysate into isolated energized *N. crassa* mitochondria. Reticulocyte lysate containing the ^{35}S-labeled preproteins was incubated with isolated mitochondria for 6 min at 25°C (this is within the kinetically linear import time). In the case of the samples for panels B and C, the mitochondria were treated with proteinase K (100 μg/ml). The mitochondria were reisolated, and the proteins were analyzed by SDS-PAGE, fluorography, and densitometry and with a storage phosphor imaging system (Molecular Dynamics). The amount of protein imported into wild-type mitochondria was set at 100%. The precursor of the ADP/ATP carrier does not contain a cleavable presequence. (D) Import of unfolded Su9-DHFR into energized mitochondria. Urea-denatured Su9-DHFR was diluted 40-fold into the import reaction (39). An incubation at 25°C was performed for the times indicated. Import was analyzed as described for panel C. To dissipate the membrane potential (Δψ), 8 μM antimycin A, 20 μM oligomycin, and 0.5 μM valinomycin were included (39, 56). The amount of Su9-DHFR imported into WT mitochondria within 6 min was set to 100%. Δψ, membrane potential across the mitochondrial inner membrane; WT, wild-type mitochondria; *csr-1*, mitochondria lacking CyP20; p, i, and m, precursor-, intermediate-, and mature-sized forms of Su9-DHFR, respectively.

efficient import into *csr-1* mitochondria demonstrates that the lack of CyP20 neither interferes with the generation of a Δψ nor blocks the function of these essential matrix proteins.

Folding of Su9-DHFR is delayed in mitochondria lacking CyP20. On the basis of the effect of CsA in the folding of imported Su9-DHFR, we compared the rates of folding in mitochondria isolated from the wild-type strain and in mitochondria isolated from the *csr-1* mutant. pSu9-DHFR was imported from urea, and after 30 s import was stopped by dissipation of the Δψ. After different periods of further incubation at 25°C, the mitochondria were cooled to 0°C, treated with protease, and reisolated. The folding state of DHFR molecules was determined by treatment with proteinase K after lysis of the mitochondria in detergent. In wild-type mitochondria, about 50% of the molecules were folded after 5 min, and 80 to 90% of the imported molecules were folded within 10 min (Fig. 5A). When the membrane potential was dissipated before the addition of preprotein, no protease-resistant DHFR was observed (Fig. 5A, right lanes), excluding the possibility that folding of nonimported molecules was observed. In *csr-1* mitochondria, folding of DHFR was strongly delayed, reaching about 50% folded molecules after 20 min. When the reaction was performed at a lower temperature (10°C), folding in wild-type mitochondria occurred at a slower rate (Fig. 5B). The folding in *csr-1* mitochondria was again considerably retarded. The lack of CyP20 thus delays the folding of proteins imported into mitochondria.

We asked if the inhibitory effect of CsA on folding of mSu9-DHFR depends on CyP20. The folding reaction in *csr-1* mitochondria was assayed in the presence and in the absence of CsA. As shown in Fig. 6, CsA did not affect (the slow) protein folding in the matrix of the mutant mitochondria, whereas the same concentrations of CsA significantly delayed folding in wild-type mitochondria (Fig. 1B). CyP20 is obviously the major target of CsA in its effect on the refolding of imported Su9-DHFR.

In the absence of CyP20, imported Su9-DHFR accumulates at Hsp70 and Hsp60. Does the lack of CyP20 influence the interaction of imported protein molecules with chaperones in the mitochondrial matrix? Imported proteins transiently interact with matrix Hsp70 of wild-type mitochondria, as demonstrated by coimmunoprecipitation with mt-Hsp70-specific antibodies (Fig. 7A) (32). No coimmunoprecipitation was observed with preimmune sera (Fig. 7A). In *csr-1* mitochondria, the association of imported DHFR with mt-Hsp70 was enhanced and prolonged with a broad peak at 5 to 10 min. The sensitivity to treatment with proteinase K demonstrated that

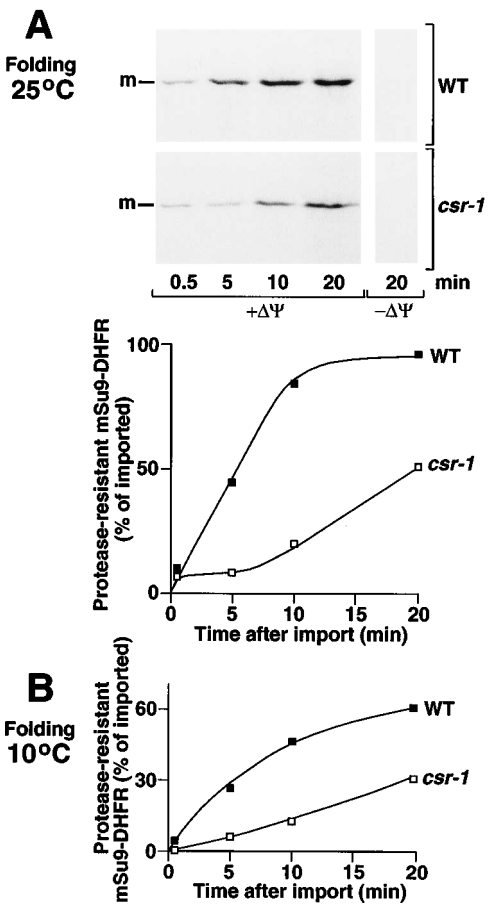

FIG. 5. Refolding of an imported protein is impaired in mitochondria lacking cyclophilin. (A [upper panel]) Folding of urea-denatured imported Su9-DHFR in mitochondria at 25°C. Su9-DHFR was imported into isolated mitochondria in the presence or absence of a $\Delta\psi$ as described in the legend to Fig. 4D. After 30 s, the import reaction was stopped by the addition of valinomycin, antimycin A, and oligomycin. The samples were further incubated at 25°C for the times indicated to allow folding of the imported preprotein (39). After treatment with trypsin and reisolation of the mitochondria, the samples were split into halves, and one half of each sample was treated with proteinase K. Subsequently, all samples received 1% Triton X-100 for lysis of the mitochondria. The samples were shaken at 4°C for 2 min and then incubated on ice for 8 min. All samples were treated with PMSF, and then the proteins were precipitated with TCA. The samples were analyzed as described in the legend to Fig. 4. Shown is mSu9-DHFR (m) resistant to treatment with proteinase K. The total amount of imported mSu9-DHFR (protected against treatment of mitochondria with trypsin [39]) was set at 100%. $+\Delta\psi$, membrane potential present during the import reaction; $-\Delta\psi$, dissipation of the membrane potential for the import reaction. (A [lower panel]) Quantitation of fluorographs by 2D densitometry. Shown is imported and processed Su9-DHFR (mSu9-DHFR) that was resistant to treatment with proteinase K after lysis of mitochondria. Times indicate the folding times after the import reaction. WT, wild-type *N. crassa* mitochondria; *csr-1*, *N. crassa* mitochondria lacking cyclophilin. (B) Refolding at 10°C. The experiment was performed as described above except that incubations for import and refolding were performed at 10°C.

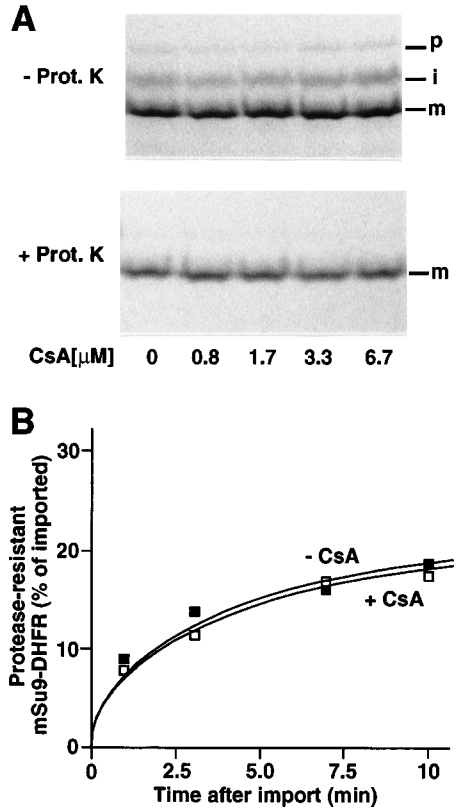

FIG. 6. Import and folding of pSu9-DHFR in *csr-1* mitochondria in the presence of CsA. (A) Test of different concentrations of CsA. (A [upper panel]) Mitochondria were isolated from the *csr-1* strain and preincubated with increasing concentrations of CsA. pSu9-DHFR was imported from 8 M urea at 25°C for 30 s. The import reaction was stopped by the addition of valinomycin, antimycin A, and oligomycin. After 10 min of further incubation at 25°C, the samples were cooled to 0°C, reisolated, and treated with trypsin. The mitochondria were then analyzed by SDS-PAGE and fluorography. (A [lower panel]) pSu9-DHFR was imported into *csr-1* mitochondria as described above. Then the mitochondria were lysed in detergent and treated with proteinase K (Prot. K; 100 μg/ml) for 10 min at 0°C. The samples were treated with PMSF and were precipitated with TCA. (B) Kinetics of refolding. *csr-1* mitochondria were preincubated in the presence or absence of 5 μM CsA. Import of pSu9-DHFR was stopped after 30 s, and the degree of intramitochondrial refolding at 25°C was determined by resistance against proteinase K. The samples were precipitated with TCA and analyzed by SDS-PAGE, fluorography, and densitometry. The quantitations show the percents proteinase K-resistant protein relative to the total amounts of imported protein.

the Hsp70-bound DHFR molecules were in a nonnative (unfolded) conformation (Fig. 7A).

Following interaction with mt-Hsp70, imported proteins are usually transferred to Hsp60, where the refolding takes place (13, 17, 22, 39, 59). In the case of wild-type mitochondria, prolonged association with Hsp60 can be monitored by coimmunoprecipitations when the folding process is delayed, for example, by lowering the levels of ATP or by addition of ATP analogs (39). In the presence of matrix ATP, however, only small amounts of imported proteins are found associated with Hsp60 as measured by coimmunoprecipitation (Fig. 7B) (35). In contrast, in mitochondria of the *csr-1* mutant we found a large amount of mSu9-DHFR associated with Hsp60 (Fig. 7B). The *csr-1* matrix contained sufficient levels of ATP, as the Hsp70-driven protein import is highly sensitive to lowering of matrix ATP levels (18, 60). In agreement with earlier observations (26, 35), the kinetics of association with Hsp60 were delayed compared with those of association with Hsp70. Hsp60-bound DHFR was again in a nonnative conformation (data not shown). The lack of CyP20 thus strongly enhances and prolongs the association of the preprotein with the two mitochondrial chaperone systems Hsp70 and Hsp60.

Nonnative Su9-DHFR molecules accumulating in CyP20-deficient mitochondria are folding competent. We asked if the nonnative mSu9-DHFR molecules, which were accumulated at Hsp70 and Hsp60 in the matrix, were irreversibly denatured or aggregated or if they represented folding intermediates which were competent for correct folding. The second possibility

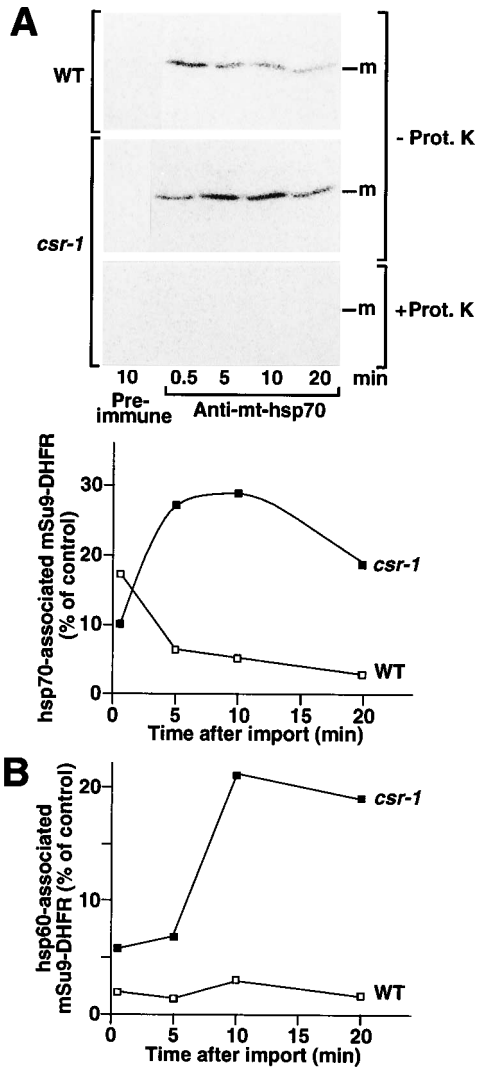

FIG. 7. The imported protein accumulates at Hsp70 and Hsp60 in cyclophilin-deficient mitochondria. (A [upper panel]) Time course of the association of mSu9-DHFR (m) with Hsp70 in mitochondria of the wild-type strain (WT) and the cyclophilin-deficient mutant (*csr-1*). Su9-DHFR was imported into mitochondria as described in the legend to Fig. 5. After dissipation of the membrane potential, the mitochondria were incubated for the indicated periods. After treatment with trypsin and reisolation, the mitochondria were lysed in Triton X-100 buffer and incubated with antibodies (from preimmune serum or directed against mt-Hsp70 or DHFR) prebound to protein A-Sepharose. The precipitated proteins were analyzed as described in the legend to Fig. 1. Protease sensitivity of the Hsp70-bound protein was assayed by treatment with 25 μg of proteinase K (Prot. K) per ml at 0°C. (A [lower panel]) Quantitation of Hsp70-associated mSu9-DHFR by 2D densitometry. Shown is the amount of mSu9-DHFR coprecipitated with antibodies directed against mt-Hsp70. The amount of mSu9-DHFR precipitated with antibodies against DHFR was set at 100%. (B) Time course of the association of mSu9-DHFR with Hsp60. The experiment was performed as described above except that antibodies against Hsp60 were used instead of anti-Hsp70 antibodies.

appeared to be more likely, since the folding in *csr-1* mitochondria was delayed but not completely blocked (Fig. 5). To directly test this possibility, we made use of the observation that Mg-ATP promotes the release of polypeptides from Hsp70 and Hsp60 and thereby allows rapid folding of DHFR in diluted samples with low protein concentrations (22, 47, 62). We thus determined if folding of imported mSu9-DHFR was possible in extracts prepared from *csr-1* mitochondria. After lysis

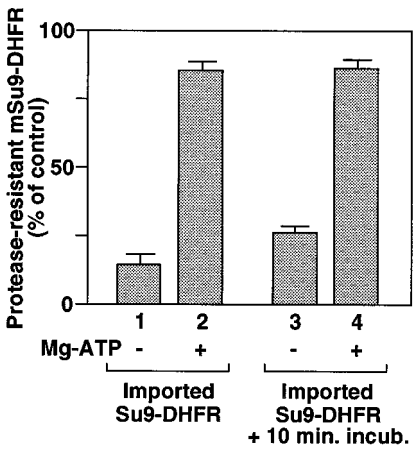

FIG. 8. Refolding of imported pSu9-DHFR after lysis of mitochondria. Su9-DHFR was imported into mitochondria of the cyclophilin-deficient strain *csr-1* as described in the legends to Fig. 4 and 5. The samples were cooled on ice either directly after import (samples 1 and 2) or after an additional incubation at 25°C for 10 min (samples 3 and 4), followed by a treatment with trypsin. The mitochondria were reisolated. After lysis in 1% Triton X-100–100 mM NaCl–50 mM KCl–10 mM Tris-HCl (pH 7.2), with the addition of either 5 mM EDTA (lanes 1 and 3) or 5 mM Mg-ATP and 7.5 μM purified CyP20 (lanes 2 and 4), the samples were treated with 100 μg of proteinase K per ml for 6 min. The total amount of imported mSu9-DHFR was set at 100%. The standard errors of the means were calculated from results of four independent experiments.

of the mitochondria, we added Mg-ATP and purified cyclophilin and assayed for protease resistance of DHFR. Columns 2 and 4 of Fig. 8 show that efficient folding indeed took place. We conclude that nonnative DHFR accumulated in *csr-1* mitochondria represents a productive intermediate competent for folding.

DISCUSSION

We have characterized the role of the mitochondrial cyclophilin CyP20 in the import and folding of proteins. Two different approaches were combined, i.e., inhibition of the PPIase activity of CyP20 with the drug CsA and characterization of a CyP20-deficient mutant. We found that CyP20 is not required for the translocation of precursor proteins across the mitochondrial membranes and for proteolytic cleavage of their presequences in the matrix. To monitor the kinetics of protein folding in the matrix, we used a model protein that acquires high protease resistance only in its correctly folded conformation. Folding of the model protein was three- to fourfold slower in CyP20-deficient (*csr-1*) mitochondria than in wild-type mitochondria. It has to be emphasized that the lack of CyP20 did not block protein folding in the matrix, but only delayed the folding process. CsA had an inhibitory effect only on refolding in mitochondria of the wild-type strain, and not on refolding in those of the *csr-1* mutant. Refolding experiments with isolated CyP20 in vitro confirmed that the model protein is a substrate of the cyclophilin. We conclude that CyP20 is a component of the protein folding machinery in the mitochondrial matrix.

The protein molecules, which were delayed in folding in the matrix of *csr-1* mitochondria, were not freely soluble in the matrix but were associated with molecular chaperones. Their association with the heat shock proteins Hsp70 and Hsp60 was considerably prolonged in comparison with their association with these proteins in wild-type mitochondria. An essential difference between the diluted in vitro systems and the mitochondrial matrix is the protein concentration, which is very

high in the matrix. Molecular chaperones protect nonnative molecules from aggregation, misfolding, and nonproductive interactions (12, 17, 22). In the absence of cyclophilin, the folding of imported proteins is retarded, and the reversible accumulation of the nonnative molecules at Hsp70 and Hsp60 thus seems to function as a safety system to minimize nonproductive side reactions in the folding pathway. It has been shown that folding processes require many cycles of binding to and release from the chaperones (8, 22, 51, 62, 69). In ATP-dependent reactions, polypeptides are released from the chaperones, and depending on their folding state, they either fold correctly or rebind to the chaperones. Released preproteins that require proline isomerization interact with PPIases, which accelerate isomerization-dependent slow refolding reactions. Thus, PPIases reduce the number of cycles required on the way to a productive folding of a polypeptide.

As the *N. crassa* gene for CyP20 encodes both the cytosolic form and the mitochondrial form, the mutant *csr-1* cells lack both CyP20 isoforms (65). The following evidence excludes the possibility that the lack of these two cyclophilins indirectly affects protein folding inside mitochondria, for example, by blocking the biogenesis or assembly of matrix heat shock proteins. (i) The mutant mitochondria are not impaired in the generation of a membrane potential ($\Delta\psi$), as the import of preproteins, which strictly depends on a $\Delta\psi$ (41), is not affected. (ii) The import of preproteins into the matrix fully depends on functional matrix Hsp70 and a sufficient level of ATP (16, 18, 60). The efficient import of preproteins demonstrates that these two parameters also are not impaired in the mutant mitochondria. (iii) Proteolytic processing of imported proteins occurs with wild-type efficiency, demonstrating that two further essential matrix proteins, the two subunits of the mitochondrial processing peptidase, are functional. (iv) The Hsp60 of the mutant mitochondria is functional in protein folding in an ATP-dependent manner. (v) The effect of CsA on isolated wild-type mitochondria shows the same selectivity as the lack of CyP20, that is, a delay of folding in the matrix without impairment of the membrane translocation of preproteins, and CsA has no effect in the mutant mitochondria. The combined evidence thus strongly suggests that the observed delay in folding of imported Su9-DHFR in CyP20-deficient mitochondria is selectively caused by the lack of matrix CyP20.

At a first glance it may seem surprising that *N. crassa* cells lacking CyP20 are viable and retain mitochondrial functions such as respiration and protein import. We consider the buffer function of chaperones and the redundancy of the PPIases the most likely explanations for the fact that *csr-1* cells are only slightly reduced in their growth rates (28). With regard to buffer function, molecular chaperones serve as a buffer system to keep nonnative molecules in a folding-competent state. Thus, protein folding in the matrix of cyclophilin-deficient mitochondria is delayed but not completely blocked. In contrast, mutants of the chaperones Hsp70 and Hsp60 were found to block folding in the matrix, and a deletion of either chaperone was lethal (8, 26). With regard to redundancy, two large families of PPIases are known to exist: cyclophilins and FK506-binding proteins (30, 52). A third family of prolyl isomerases, called parvulins, has been discovered only recently (43). It has been demonstrated that different PPIases have distinct but overlapping substrate specificities (21) and that they may thus provide a partial complementation of each other's function. In fact, at least one additional PPIase, an FK506-binding protein, has been determined to be present in the mitochondrial matrix of *N. crassa* (28). Studies of *S. cerevisiae* similarly suggest a redundancy of the system of PPIases in various cellular compartments (11, 61). Deletion of a putative yeast mitochondrial cyclophilin led to a mitochondrion-related phenotype, i.e., a selective inability to grow on lactate at 37°C, suggesting a possible involvement of this cyclophilin in the maturation of cytochrome b_2 (11). At 30°C, however, this deletion mutant lacked any phenotype (11). The observation that a cellular protein is not essential for the viability of cells may suggest that its function is not of general importance for cell functions, or alternatively, it may indicate that its function is of such great importance that additional components performing similar functions are present in the cell (functional redundancy). In the case of mitochondrial protein biogenesis, the latter possibility was, for example, observed for the mitochondrial import receptors (20, 36, 44) and the cytosolic Hsp70s (12). Since PPIases form families of highly abundant, ubiquitous, and evolutionarily conserved cellular proteins, it is suggested that a high level of redundancy of their functions is required for cell viability. Future studies will have to address the role of further PPIases in mitochondria and analyze the folding pathways of distinct preproteins (to date, however, Su9-DHFR is the only preprotein that allows a reliable and sensitive assay to assess folding after efficient import into isolated mitochondria). Reports concerning the involvement of cyclophilins in the tolerance of yeast cells against heat stress have suggested a function of cyclophilins in maintaining the native folding state of cellular proteins (11, 61). Combined genetic and electron microscopic studies of *NinaA* mutants of *D. melanogaster* (9, 38, 54,

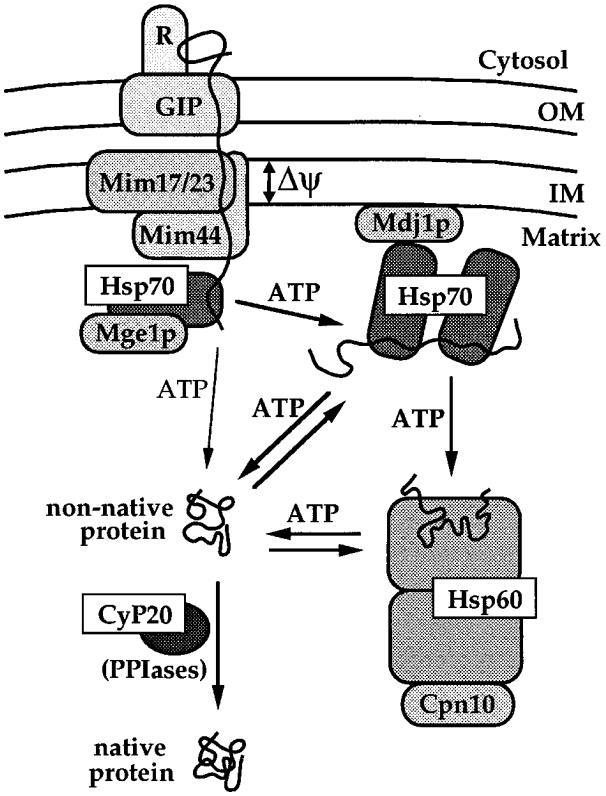

FIG. 9. Hypothetical model for mitochondrial import and folding of a preprotein requiring *cis-trans* prolyl isomerization in the matrix. See the text for details. Cpn10, chaperonin 10; CyP20, 20-kDa cyclophilin; $\Delta\psi$, membrane potential; GIP, general insertion pore; Hsp70 and Hsp60, 70- and 60-kDa heat shock proteins, respectively; IM, inner membrane; Mdj1p, mitochondrial DnaJ; Mge1p, mitochondrial GrpE; Mim, mitochondrial inner membrane import machinery; OM, mitochondrial outer membrane; R, receptor. PPIases may similarly interfere with folding reactions of proteins after release from ribosomes or after translocation across other membranes.

57) and intracellular effects of CsA (23, 32, 58) have indicated that immunophilins are involved in protein transport and maturation processes in the secretory pathway. Together with the experiments described here, this suggests a role for PPIases in chaperone-mediated protein folding processes in the cell. Mitochondrial CyP20 and other abundant single-domain PPIases in the cell (e.g., FKBP13 [66]) mainly function as soluble catalysts of protein folding (27), while less abundant larger immunophilins which contain additional domains (like NinaA or FKBP52) may be specialized in interaction with a subset of proteins (24, 30) and may be able to function as molecular chaperones themselves (2).

Figure 9 summarizes our working model of the role of molecular chaperones and single-domain PPIases in protein translocation and folding in the mitochondrial matrix, based on the findings reported here and in previous reports (summarized in references 22, 40, and 59). The preprotein is translocated through the protein import machineries of the mitochondrial outer membrane and inner membrane. mt-Hsp70 complexed with Mge1p is prebound to the inner membrane protein Mim44, directly binds to the incoming polypeptide chain, and is released from Mim44. The membrane potential ($\Delta\psi$) and conformational changes accompanying the nucleotide-dependent release of mt-Hsp70 provide the driving force for membrane translocation. Mge1p is released from mt-Hsp70 and, in a process involving Mdj1p, the preprotein is transferred to Hsp60. In ATP-dependent processes, the preproteins are released from Hsp70 or Hsp60 and most of them rebind to the chaperones, as their folding state is not sufficient to allow folding to the native conformation (recent data indicate that Hsp60-mediated folding occurs in an all-or-none manner; after rebinding, a polypeptide seems to have the same structure as it had in the original complex [69]). After each cycle of release, a small fraction of the protein is folded to its native conformation. PPIases accelerate the slow refolding processes of those released proteins that require peptidyl-prolyl isomerization. A lack of or reduced activity of PPIases retards the folding and thus favors rebinding to the chaperones. Since every binding-release cycle of Hsp70 and Hsp60 requires the hydrolysis of additional ATP (22), we propose that PPIases function in saving metabolic energy by increasing the rate of folding of some proteins.

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