Cyclophilin catalyzes protein folding in yeast mitochondria

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ABSTRACT Cyclophilins are a family of ubiquitous proteins that are the intracellular target of the immunosuppres-
sant drug cyclosporin A. Although cyclophilins catalyze peptide/prolyl cis–trans isomerization in vitro, it has remained open whether they also perform this function in vivo. Here we show that Cpr3p, a cyclophilin in the matrix of yeast mitochondria, accelerates the refolding of a fusion protein that was synthesized in a reticulocyte lysate and imported into the matrix of isolated yeast mitochondria. The fusion protein consisted of the matrix-targeting sequence of subunit 9 of F\textsubscript{1}F\textsubscript{0}-ATPase fused to mouse dihydrofolate reductase. Refold-
ing of the dihydrofolate reductase moiety in the matrix was monitored by acquisition of resistance to proteinase K. The rate of refolding was reduced by a factor of 2–6 by 2.5 μM cyclosporin A. This reduced rate of folding was also observed with mitochondria lacking Cpr3p. In these mitochondria, protein folding was insensitive to cyclosporin A. The rate of protein import was not affected by cyclosporin A or by deletion of Cpr3p.

Cyclophilins are a large family of highly conserved proteins. The cyclophilins found in eukaryotic cells tightly bind cyclo-
sporin A, an undecameric cyclic peptide. In vertebrates, the cyclophilin–cyclosporin A complex suppresses the immune response because it inhibits the calmodulin-dependent phos-
phatase calcineurin and thereby prevents the translocation of a subunit of a T cell-specific transcription factor from the cytosol to the nucleus (1). In vitro, cyclophilins also catalyze the isomerization of prolyl peptide bonds (2, 3). Although this function of cyclophilins is inhibited by cyclosporin A, the inhibition appears to be unrelated to immunosuppression because some drugs inhibit the isomerization activity of cyclophi-
lims without being immunosuppressive (4). The physiological function of cyclophilins is thus not well understood. Several reports implicate cyclophilins in protein folding in vivo. First, the cyclophilin homolog ninaA is required for the secretion of Rh1 opsin in Drosophila photoreceptor cells (5) and forms a stable complex with this protein. Second, human cyclophilin A is found to be associated with human immunodeficiency virus type 1 (HIV-1) virions, and this interaction appears to be specific for a single proline residue in the Gag polyprotein (6–8). Finally, cyclosporin A inhibits collagen folding in chicken embryo tendon fibroblast suspensions (9). However, in none of these studies was a direct involvement of cyclophilins in protein folding conclusively shown because protein folding was not measured directly or because indirect effects of cyclosporin A could not be ruled out.

The yeast Saccharomyces cerevisiae contains at least five cyclophilins (1, 10). One of these, Cpr3p, is found in the mitochondrial matrix (11). Yeast lacking Cpr3p are unable to grow at 37°C with l-lactate as the major energy source, indicating that Cpr3p is required for mitochondrial metabo-
llism at elevated temperatures (11).

In an attempt to investigate a possible function of cyclophi-
lims in intracellular protein folding, we have studied the effect of Cpr3p on the refolding of a newly imported mitochondrial precursor protein. Most mitochondrial proteins are synthe-
sized in the cytosol and then transported into mitochondria. Those destined for the mitochondrial matrix pass through proteinaceous channels spanning the outer and inner membrane and are pulled into the matrix by the ATP-dependent action of mitochondrial 70-kDa heat shock protein (hsp70) and its cochaperone GrpEp (12–14). The proteins must at least partly unfold during transport (15) and must then refold in the matrix compartment. The slowest step in the folding of small proteins is frequently the isomerization of peptide bonds preceding proline residues (16, 17). Thus, it is an attractive hypothesis that Cpr3p catalyzes the refolding of mitochondrial proteins in the matrix. Here we show that this is indeed the case.

MATERIALS AND METHODS

Precursor Protein and Mitochondria. The precursor protein used in this paper, Su9-DHFR, is a fusion of the first 69 residues of subunit 9 of Neurospora crassa F\textsubscript{1}F\textsubscript{0}-ATPase to mouse dihydrofolate reductase (DHFR) (18). All experiments described here were performed with the radioactive fusion protein that had been expressed from an SP6 promoter by in vitro transcription and translation in a rabbit reticulocyte lysate containing [\textsuperscript{35}S]methionine (19). After translation, the pre-
cursor preparation was depleted of ATP by incubation with 70 units of apyrase per ml for 5 min at 30°C, denatured with 8 M urea/25 mM Tris, pH 7.4/25 mM dithiothreitol, and incubated at room temperature for 10–30 min as described (20).

Mitochondria were isolated from Saccharomyces cerevisiae strains D273-10B [Mata, ATCC 25657 (21)], JK9-3d [MAT\textalpha, leu2, ura3, rme1, trp1, his3\textalpha, GAL\textsuperscript{+}, (11)], and ED80-2\textsuperscript{su} [MAT\textalpha, leu2, ura3, rme1, trp1, his3\textalpha, GAL\textsuperscript{+}, cpr3::HIS3 (11); hereafter referred to as strain Δcpr3] and purified on Nycodenz gradi-
ents (22). Strains D273-10B and JK9-3d contain wild-type Cpr3p. Strain Δcpr3 is isogenic to JK9-3d except that its CPR3 gene has been disrupted by insertion of the HIS3 gene (11).

Import and Folding Assays. To deplete intramitochondial ATP, mitochondria were preincubated at 0.5 mg of mitochondrial protein per ml in 3.95 ml of import buffer (final pH = 7.0; 0.6 M sorbitol/50 mM Hepes/50 mM KCl/10 mM MgCl\textsubscript{2} 2 mM KH\textsubscript{2}PO\textsubscript{4}/5 mM methionine/1 mg of fatty acid-free bovine serum albumin per ml) containing 10 units of apyrase, 5 μg of oligomycin, and 2 μg of efrapeptin per ml for 5 min on ice. To establish a membrane potential, NADH was added to 1 mM, and incubation was continued for 5 min at 25°C. An aliquot (50 μl) of denatured precursor protein was then added to the import mixture with mixing, additional NADH was added to increase the final NADH concentration to 3 mM, and incubation was continued for 10 min at 20°C. Precursor bound to the mitochondrial surface was then digested by incubation with trypsin at 100 μg/ml on ice for 45 min. Trypsin was inhibited

Abbreviations: DHFR, dihydrofolate reductase; hsp, heat shock pro-
tein.

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were added and analyzed with mitochondria from yeast strain JK9-3d. The graphs show quantitations of fluorograms of import and folding experiments as described in Materials and Methods. The extent of folding and import is given as a percentage of the total amount of precursor accumulated in the mitochondria.

with trypsin inhibitor at 200 μg/ml and the mitochondria were reisolated by centrifugation for 5 min at 8000 × g, resuspended in 2.7 ml of import buffer containing 200 μg of trypsin inhibitor, 5 μg of oligomycin, and 2 μg of efrapeptin per ml and split into two 1.08-ml aliquots. Cyclosporin A or SDZ NIM811 [3.25 μl of a stock solution in tetrahydrofuran containing 0.47 M LiCl (23)] was added to a final concentration of 2.5 μg/ml to one aliquot, and the same volume of tetrahydrofuran containing 0.47 M LiCl was added to the other. Both aliquots were incubated for 5 min on ice and then for 5 min at 30°C. The ATP-depletion intermediate generated by this procedure was then chased into the matrix by addition of an ATP-regenerating system (2 mM ATP/5 mM α-ketoglutarate/10 mM creatine phosphate/1.5 mg of creatine kinase per ml), and the mitochondria were kept at 30°C throughout the remainder of the experiment. Samples (100 μl) were taken before adding the ATP-regenerating system and at various times thereafter and were analyzed for imported or folded 35S-labeled DHFR.

To assay for folded DHFR, samples from the import mixture were mixed with an equal volume of ice-cold import buffer containing 2% Triton X-100 and 200 μg of proteinase K per ml but no bovine serum albumin. Treatment of folded DHFR with proteinase K yields a protease-resistant 21-kDa fragment, whereas unfolded DHFR yields fragments that are too small to be detected by SDS/PAGE. After incubation for 30 min on ice, phenylmethylsulfonyl fluoride was added to 1 mM, proteins were precipitated with 5% trichloroacetic acid, and the samples were analyzed by SDS/PAGE with a Tricine-based buffer system (24). To analyze protein import, the samples were added to 7 volumes of "mitoisolating buffer" (20 mM Hepes, adjusted to pH 7.4 with KOH/1 mg of fatty acid-free bovine serum albumin per ml) containing 100 μg of proteinase K per ml and incubated for 30 min on ice. This procedure disrupts the outer mitochondrial membrane but leaves the inner membrane intact (20). After addition of phenylmethylsulfonyl fluoride to 1 mM, the mitoplasts were reisolated by centrifugation (5 min at 8000 × g), resuspended in import buffer without bovine serum albumin, precipitated with 5% trichloroacetic acid, and analyzed by SDS/PAGE. For determining the total amount of precursor accumulated in the mitochondria at the end of the experiment, a sample was taken after the last time point and precipitated with 5% trichloroacetic acid. Folding and import were analyzed by fluorography of the dried gels and quantification of the fluorograms with a computing densitometer (model 300A, Molecular Dynamics).

Table 1. Rate constants (k; ±SE) for folding of the imported DHFR domain at 30°C in the absence and presence of cyclosporin A (CsA) at 2.5 μg/ml after import into mitochondria from different wild-type yeast strains (JK9-3d and D273-10B) or from a mutant lacking a functional CPR3 gene (Δcpr3)

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Without CsA (2.5 μg/ml)</th>
<th>With CsA</th>
<th>Ratio k-CsA/k-Δcpr3</th>
</tr>
</thead>
<tbody>
<tr>
<td>D273-10B</td>
<td>0.9 ± 0.2</td>
<td>0.15 ± 0.01</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>JK9-3d</td>
<td>0.43 ± 0.09</td>
<td>0.16 ± 0.02</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>Δcpr3</td>
<td>0.35 ± 0.04</td>
<td>0.15 ± 0.02</td>
<td>2.3 ± 0.4</td>
</tr>
</tbody>
</table>

FIG. 1. Import of the ATP-depletion intermediate is much more rapid than its refolding in the matrix. The experiment was performed with mitochondria from yeast strain JK9-3d. The graphs show quantitations of fluorograms of import and folding experiments as described in Materials and Methods. The extent of folding and import is given as a percentage of the total amount of precursor accumulated in the mitochondria.

FIG. 2. Folding of the imported DHFR domain in the matrix of mitochondria is inhibited by cyclosporin A. (Top) Fluorograms depicting the acquisition of protease-resistance of DHFR in mitochondria in the presence (●) and absence (○) of cyclosporin A (CsA) at 2.5 μg/ml. The experiment was performed with mitochondria from yeast strain JK9-3d. Samples were taken at the indicated times and treated with Triton X-100 and proteinase K. The lane marked "total" contains a sample precipitated with 5% trichloroacetic acid. (Middle) Quantitation of the experiment shown in Top. The extent of folding is plotted as a percentage of the total amount of protein accumulated in the mitochondria at the end of the experiment. (Bottom) Same as Middle except that the experiment was performed with mitochondria from yeast strain D273-10B.
The extent of import or folding was plotted as a percentage of the total amount of precursor accumulated in the mitochondria at the end of the time course. The kinetics of folding and import were analyzed assuming a simple first-order process and fitting to the equation

\[ A = A_0 \left[ 1 - \exp(-kt) \right] + C, \]

where \( A \) is the extent of import or folding at any given time, \( A_0 \) is the total amount of import or folding, \( k \) is the rate constant of import or folding, and \( C \) is a constant offset due to the background of the film.

**Construction of C-Terminally Hexahistidine-Tagged Cpr3p.** The CPR3 gene was amplified from genomic DNA of yeast strain JK9-3d by the polymerase chain reaction with Pfu DNA polymerase (Stratagene), and the amplified product was cloned into plasmid pQE60 (Qiagen, Chatsworth, CA) by using unique EcoRI and BamHI sites in the plasmid. This construction added 10 amino acids to the C terminus of Cpr3p to yield the C-terminal sequence Gly-Ser-Arg-Ser-His-His-His-His-His-His-His. The construct was then cloned into the yeast- *Escherichia coli* shuttle vector pGR401 (gift from G. Reid, University of Edinburgh, Edinburgh, U.K.) by using unique EcoRI and HindIII sites. The new construct, termed pAM21A, carried the tagged CPR3 gene under the control of the alcohol dehydrogenase I promoter, the yeast URA3 gene, a gene encoding \( \beta \)-lactamase, the replication origin of the 2-\( \mu \)m yeast plasmid, as well as the replication origins fl and CoIE1. When the yeast strain ED80-2 was transformed with this plasmid, its growth defect in lactate medium at elevated temperatures was overcome, indicating that the tagged Cpr3p was expressed and functional.

**Purification of Hexahistidine-Tagged Cpr3p.** Mitochondria were purified from Acpr3p cells transformed with the plasmid pAM21A. An aliquot (0.4 mg) of mitochondria in 0.4 ml of solubilization buffer (150 mM NaCl/50 mM Heps, pH 7.4/10% (vol/vol) glycerol/20 mM imidazol) was disrupted by freezing in liquid nitrogen and thawing in a sonicating water bath at room temperature. The suspension was centrifuged for 15 min at 15,000 \( \times \) g, and the supernatant was incubated for 1 hr at 4°C with 25 \( \mu \)l of a slurry containing equal volumes of Ni-nitrilotriacetic acid (NTA)-agarose (Qiagen) and water. The beads were then washed four times with solubilization buffer, heated to 95°C in SDS-containing gel-loading buffer (24), and analyzed by SDS/PAGE.

**Chemicals.** Apyrase (grade VIII), trypsin (grade XIII), and trypsin inhibitor (type I-S) were obtained from Sigma, and proteinase K was from Boehringer Mannheim. D-Sorbitol (Baker grade) was from J.T. Baker, and cyclosporin A and SDZ NIM811 were gifts from Sandoz Pharmaceutical.

### RESULTS

When urea-denatured Su9-DHFR was incubated with ATP-depleted mitochondria that still maintained an electrochemical potential across their inner membrane, import of the precursor into the matrix was arrested. In the resulting "ATP-depletion intermediate," the presequence had already inserted across the inner membrane, but most of the precursor chain was in the intermembrane space between the two mitochondrial membranes (25). Upon addition of an ATP-regenerating system, the translocation-arrested Su9-DHFR was rapidly imported into the matrix, where it refolded to a protease-resistant form. Import into the matrix was at least 2.5 times faster than folding (Fig. 1).

Addition of cyclosporin A to 2.5 \( \mu \)g/ml to mitochondria from two different wild-type yeast strains (JK9-3d and D273-10B) inhibited the refolding rate of DHFR in the matrix by 50–85% (Table 2 and Fig. 2) but did not affect the rate of import (Table 2 and Fig. 3). When the same experiment was done with mitochondria from Acpr3p yeast which lack the matrix-located cyclophilin Cpr3p, cyclosporin A did not inhibit folding of the imported DHFR. In these mitochondria, DHFR refolded at the same slow rate seen with wild-type mitochondria in the presence of cyclosporin A (Table 1 and Fig. 4 *Upper*). Deletion of the mitochondrial cyclophilin had no effect on the rate at which Su9-DHFR was imported (Table 2 and Fig. 4 *Lower*).

Is catalysis of protein folding in mitochondria by cyclophilin caused by the peptidylproline isomerase activity of cyclophilin or does it reflect some other mechanism? To distinguish these two possibilities, we studied the effect of a second compound, SDZ NIM811, on the folding of imported DHFR in wild-type and cyclophilin-deficient mitochondria. This compound lacks immunosuppressive activity, yet inhibits the peptidylprolyl cis-trans isomerase activity of cyclophilin as potently as cyclosporin A does (8). Fig. 5 shows that in wild-type mitochondria, SDZ NIM811 inhibited protein folding to the same extent as cyclosporin A did, whereas it had no effect on DHFR folding in cyclophilin-deficient mitochondria.

In an attempt to detect a direct interaction of imported DHFR with the mitochondrial cyclophilin, we constructed a yeast strain in which all Cpr3p molecules had a C-terminal hexahistidine tail. The histidine-tagged cyclophilin appeared to be functional because DHFR imported into the corresponding mitochondria refolded at the same rate as in wild-type mitochondria, and refolding was sensitive to cyclophilin A (data not shown). When the tagged cyclophilin was affinity-purified by a one-step procedure from mitochondria disrupted by sonication, it proved to be devoid of other partner proteins. When it was purified from mitochondria before or after 0.4, 1, 2, and 10 min of chasing the ATP-depletion intermediate into the matrix, the Cpr3p was free of radiolabeled DHFR. Our method thus did not detect association of the cyclophilin with other mitochondrial proteins or with one of its substrates.

### Table 2. Rate constants (k; \( \pm SE \)) for the import of the ATP-depletion intermediate of DHFR at 30°C into mitochondria from a wild-type yeast strain (JK9-3d) or from a mutant lacking a functional CPR3 gene (Acpr3) in the absence and presence of 2.5 \( \mu \)g/ml cyclosporin A (CsA)

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Without CsA (2.5 ( \mu )g/ml)</th>
<th>With CsA (2.5 ( \mu )g/ml)</th>
<th>Ratio ( k_{-CsA}/k_{+CsA} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>JK9-3d</td>
<td>0.9 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Acpr3</td>
<td>1.5 ± 0.2</td>
<td>1.7 ± 0.3</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

**Fig. 3.** Cyclosporin A at 2.5 \( \mu \)g/ml does not inhibit import of Su9-DHFR into mitochondria. The experiment was the same as that described in Fig. 2 *Top and Middle* except that import was measured.
**DISCUSSION**

Proteins that are transported into mitochondria must unfold at least partially to cross the mitochondrial membranes and then refold at their final location within the mitochondria. Refolding of different matrix proteins is mediated by hsp60 and cpn10, the mitochondrial DnaJ homolog Mdj1p (12), and probably other mitochondrial chaperones that are still undiscovered. Here we show that the mitochondrial cyclophilin Cpr3p is yet another folding helper in the matrix space: First, specific cyclophilin ligands such as cyclosporin A inhibit refolding of DHFR in the matrix without affecting protein import. Second, deleting the mitochondrial cyclophilin Cpr3p inhibits the rate of intramitochondrial protein folding to the same extent as addition of cyclosporin A or SDZ NIM811 to wild-type mitochondria but has no effect on protein import. Third, cyclosporin A and SDZ NIM811 do not inhibit folding of DHFR in cyclophilin-deficient mitochondria. These observations show that cyclosporin A and SDZ NIM811 inhibit refolding as a result of their interaction with Cpr3p and that this inhibition is not caused by some indirect effect of a toxic cyclophilin–ligand complex. Since cyclophilins promote the refolding of proteins in vitro by catalyzing peptidylproline isomerization and two different compounds that bind to the site of isomerase activity of cyclophilin inhibit folding of DHFR in mitochondria, it is likely that Cpr3p acts as a proline isomerase in mitochondria.

Mouse DHFR contains 13 proline residues, all of them in trans configuration. In native DHFR, three of these proline residues reside within a four-residue helix in a surface loop, where they would be good substrates for cyclophilin (26). In vitro folding studies on E. coli DHFR, which is highly homologous to mouse DHFR, suggest that the isomerization of proline peptide bonds is rate-limiting for several slow folding steps (27). DHFR thus seems to be a very suitable model protein for studying the role of cyclophilins in protein folding. With other proteins, cyclophilins may have little if any effect on refolding, either because proline isomerizations are not rate-limiting or because the critical proline bonds become buried early in the refolding pathway and are thus inaccessible to the cyclophilin.

We were unable to detect a stable interaction of Cpr3p with other mitochondrial proteins or with a refolding precursor, using a rapid and gentle affinity-purification of Cpr3p. Avian cyclophilin 40 is part of the progesterone receptor multiprotein complex (28) and the Drosophila cyclophilin ninaA forms a stable complex with its substrate, Rhl opsins (5). In contrast, Cpr3p appears not to function as a stable heterooligomer; it probably only forms a classical short-lived complex with its protein substrate.

We have also tested whether an inhibition or a deletion of Cpr3p changes the interaction of precursor proteins with mitochondrial hsp70 or with the chaperonins hsp60 and cpn10. No such effect was detected. Even when refolding of DHFR was slowed down by lack of the functional cyclophilin, the imported DHFR failed to associate stably with hsp60 (S.R., unpublished observations). Refolding of DHFR under these conditions may be spontaneous or be mediated by as-yet-undiscovered mitochondrial chaperones.

Our results suggest the sequence of events outlined in Fig. 6. As the unfolded precursor protein emerges from the protein

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**FIG. 4.** Cyclosporin A at 2.5 μg/ml does not affect import or folding of Su9-DHFR in Cpr3p-deficient mitochondria. The experiment was identical to that described in Figs. 2 and 3 except that mitochondria from Δcrp3 yeast were used. (**Upper**) Folding. (**Lower**) Import.

**FIG. 5.** Compound SDZ NIM811 inhibits protein folding in wild-type mitochondria (strain JK9-3d) (**Upper**) but not in Cpr3p-deficient mitochondria (strain Δcrp3) (**Lower**). The experiment was performed as described in Fig. 2 except that SDZ NIM811 at 2.5 μg/ml was used instead of cyclosporin A.
FIG. 6. Suggested functions of mitochondrial cyclophilin Cpr3p and chaperones in import and refolding of imported proteins. OM, outer membrane; IM, inner membrane; mhs70, mitochondrial hsp70; G, GrpEp; c, cpn10.

transport channel across the inner membrane, it interacts with mitochondrial hsp70, which is the central subunit of an ATP-driven import motor (13, 14). The precursor protein is released from mitochondrial hsp70 in an incompletely folded form (25) and can then transiently interact with Cpr3p, which catalyzes isomerization of proline peptide bonds. Further experiments will be needed to determine how Cpr3p cooperates with other chaperones such as hsp60/cpn10 and whether mitochondria contain additional peptidylproline isomerases that mediate refolding of imported proteins.

During the preparation of this manuscript we learned from M. Tropschug that he and his colleagues have made similar findings with mitochondria from *Neurospora crassa* (29).

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