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Yeast Mitochondria Lacking the Phosphate Carrier/p32 Are Blocked in Phosphate Transport but Can Import Preproteins after Regeneration of a Membrane Potential

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Two different functions have been proposed for the phosphate carrier protein/p32 of *Saccharomyces cerevisiae* mitochondria: transport of phosphate and requirement for import of precursor proteins into mitochondria. We characterized a yeast mutant lacking the gene for the phosphate carrier/p32 and found both a block in the import of phosphate and a strong reduction in the import of preproteins transported to the mitochondrial inner membrane and matrix. Binding of preproteins to the surface of mutant mitochondria and import of outer membrane proteins were not inhibited, indicating that the inhibition of protein import occurred after the recognition step at the outer membrane. The membrane potential across the inner membrane of the mutant mitochondria was strongly reduced. Restoration of the membrane potential restored preprotein import but did not affect the block of phosphate transport of the mutant mitochondria. We conclude that the inhibition of protein import into mitochondria lacking the phosphate carrier/p32 is indirectly caused by a reduction of the mitochondrial membrane potential ($\Delta\gamma$), and we propose a model that the reduction of $\Delta\psi$ is due to the defective phosphate import, suggesting that phosphate transport is the primary function of the phosphate carrier/p32.

The phosphate carrier (P_iC), also termed phosphate transport protein or p32, is a 33-kDa membrane protein of *Saccharomyces cerevisiae* mitochondria. This protein has been the subject of a controversial debate in the past years.

The primary structure shows significant homology to the phosphate translocator of the mitochondrial inner membrane of mammalian mitochondria (24, 34). A characterization of mitochondria with mutant P_iC/p32 and a reconstitution of P_iC/p32 into liposomes and functional analysis strongly indicated a function of the protein in phosphate transport (12, 35, 48).

Evidence has been provided, however, that P_iC/p32 functions as an import receptor on the mitochondrial surface for nuclear-encoded preproteins (24, 27). Most mitochondrial proteins are synthesized as preproteins on cytosolic polysomes (14). They are targeted to receptor proteins on the mitochondrial surface and are subsequently translocated through specific channels of the mitochondrial outer and inner membranes (32). Protein transport across the inner membrane strictly depends on the presence of a membrane potential ($\Delta\psi$). Anti-idiotypic antibodies (which mimic mitochondrial targeting sequences) were reported to react with P_iC/p32 and to inhibit protein import into isolated mitochondria (27). Murakami et al. (25) proposed that P_iC/p32 functions primarily as a signal sequence binding subunit of a protein-conducting channel and that this channel may in addition allow transport of phosphate.

Opposing results have also been obtained for the localization of P_iC/p32 (mitochondrial outer membrane versus inner membrane), although recent results indicate that the bulk of P_iC/p32 is located in the inner membrane like the mammalian phosphate translocator (7, 9, 27, 50).

With regard to this controversy about the functional assignment of P_iC/p32, we noticed that an experimental characterization of protein import into mitochondria lacking P_iC/p32 (ΔP_iC mitochondria) had not been reported so far. We therefore analyzed ΔP_iC mitochondria and found that both phosphate transport and protein transport were inhibited. The initial suggestion that P_iC/p32 is indeed a bifunctional protein, however, could be revised by a further analysis of the mutant mitochondria. The defective protein import was caused by a reduction of the membrane potential. Restoration of a membrane potential restored protein import but did not relieve the block in phosphate transport. The defect in phosphate transport seems to lead to a reduction of the membrane potential and thus indirectly causes an inhibition of protein import. This suggests that phosphate transport is the primary function of P_iC/p32.

MATERIALS AND METHODS

***S. cerevisiae* strains.** Construction of the ΔP_iC strain by disruption of the *MIR1* gene has been described previously (7). The genotype is *MATα ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1 mir1::LEU2.* The strain W303-1B (*MATα ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1*) was used as control strain (wild type).

Isolation of mitochondria and in vitro protein import. The yeast strains were grown in YP medium (1% Bacto yeast extract, 2% Bacto Peptone, pH 5.0) containing 2% galactose. Mitochondria were isolated according to published procedures (5, 13). The mitochondrial precursor proteins were synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine (42). The import assay mixtures included isolated yeast mitochondria (5 μg of protein), 10% (vol/vol) reticulocyte lysate, 5 mM $MgCl_2$, 2 mM NADH, and bovine serum albumin (BSA) buffer (3% [wt/vol] BSA, 250 mM sucrose, 80 mM KCl, 10 mM MOPS [morpholine propanesulfonic acid], pH 7.2) in a final volume of 0.1 ml. The import reactions were carried out for 5 min at 25°C and stopped by the addition of 1 μM valinomycin. Dissipation of the membrane potential $\Delta\psi$ was performed by the addition of 1 μM valinomycin and 20 μM oligomycin prior to the import incubation. Treatment with proteinase K and analysis by sodium

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dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), fluorography, laser densitometry, and Western blotting (immunoblotting) were performed as previously described (7, 42, 49, 50).

Assessment of the mitochondrial inner membrane potential. The $\Delta\psi$ of isolated yeast mitochondria was assessed by recording the fluorescence decrease (quenching) of the voltage-sensitive dye 3,3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$; Molecular Probes] as previously described (10, 30, 39). The measurements were performed with a Perkin-Elmer 650-40 fluorescence spectrophotometer at 25°C with excitation at 622 nm, emission at 670 nm, and slits at 5 nm. The buffer used for the fluorescence measurements (1.25 ml of 0.6 M sorbitol–0.1% [wt/vol] BSA–10 mM $MgCl_2$–0.5 mM EDTA, pH 7.4) contained 3.6 μM $DiSC_3(5)$ and 80 mM KCl. The fluorescence value was set to 100 arbitrary fluorescence units, and yeast mitochondria (25 μg of protein) were injected into the cuvette to start the experiment. Further additions are reported in the legend to Fig. 4. The membrane potential across the inner membrane of mitochondria was dissipated by the addition of 2 μl of valinomycin (in ethanol; 1 μM final concentration). The difference between the fluorescences prior to and after the addition of valinomycin represents a relative assessment of the membrane potential.

Swelling of yeast mitochondria. The rate of mitochondrial swelling was monitored by recording the decrease in A_{546}, essentially as previously described (28), with a Perkin-Elmer 557 spectrophotometer. Yeast mitochondria (100 μg of protein) were added to a glass cuvette containing 1 ml of 120 mM ammonium chloride or 120 mM ammonium phosphate–20 mM Tris–1 mM EDTA–5 μM rotenone, pH 7.4.

Uptake of [^{32}P]phosphate into isolated yeast mitochondria. Phosphate transport was assayed by measuring the uptake of [^{32}P]phosphate into isolated mitochondria by the inhibitor stop method according to published procedures (29). Freshly isolated yeast mitochondria (0.25 mg of protein) were incubated at 0°C in 100 mM KCl–20 mM Tris-HCl (pH 6.5)–1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-*N*′,*N*′-tetraacetic acid]–1 μg of rotenone per ml–20 mM butylmalonate. The uptake was started by adding 1.35 mM [^{32}P]phosphate and stopped, after various times, with 1.5 mM mersalyl. After rapid centrifugation of the yeast mitochondria, the amount of [^{32}P]phosphate incorporated in the matrix space was calculated as previously described (29).

RESULTS

Mitochondria lacking P_iC/p32 are impaired in protein import. The gene *MIR1*, encoding P_iC/p32 of *S. cerevisiae* (24), has been disrupted in the *S. cerevisiae* ΔP_iC strain (7). The cells are strongly impaired in growth on a nonfermentable medium. They are able to grow on a fermentable medium (the growth rate is reduced by about one-third compared with that of wild-type cells). ΔP_iC cells had a significantly higher frequency of generation of petite colonies (complete inability to grow on nonfermentable medium due to loss of mitochondrial DNA) than the wild-type cells, as is often observed with mutants of mitochondrial proteins (19, 20). To exclude indirect effects in the analysis of protein import, such as a decrease of the membrane potential due to loss of mitochondrial DNA and inactivation of the respiratory chain, we carefully checked that the cells used had a functional mitochondrial genome, i.e., were [*rho*$^+$] (by crossing with [*rho*^{0}] tester strains) (23, 26).

ΔP_iC cells and the corresponding wild-type strain W303-1B were grown on galactose. The ΔP_iC cells accumulated uncleaved mitochondrial precursor proteins as shown for the precursor of the matrix heat shock protein Hsp60 (Fig. 1A, lane 2). We then studied in vitro import of preproteins into isolated mitochondria. The precursors of mitochondrial proteins were synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and incubated with the isolated mitochondria isolated from wild-type or mutant cells. After the import reaction, the mitochondria were treated with proteinase K to remove nonimported preproteins, reisolated, and subjected to SDS-PAGE. Three preproteins were used: the ADP/ATP carrier, which is targeted to the inner membrane (Fig. 1B, lanes 1, 2, 5, and 6); the Fe/S protein of the cytochrome bc_1 complex, which is targeted to the intermembrane space side of the inner membrane (Fig. 1B, lanes 3, 4, 7, and 8); and a fusion protein containing the presequence of *Neurospora crassa* F_0-ATPase subunit 9 and dihydrofolate reductase (Su9-DHFR), which is transported into the matrix space (Fig. 1C)

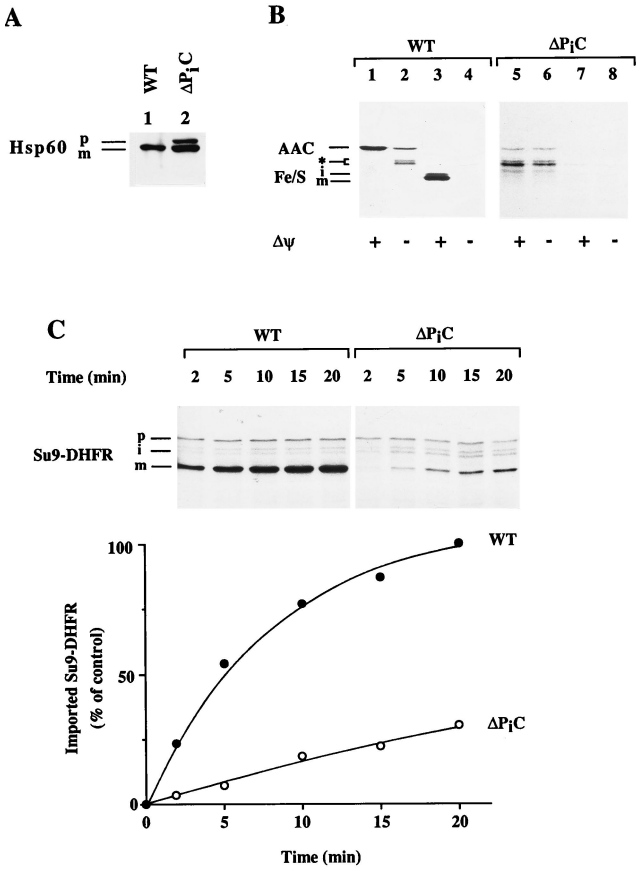

FIG. 1. Inhibition of protein import into mitochondria lacking P_iC/p32. (A) Yeast cells. Cellular extracts from the *S. cerevisiae* wild-type (WT) strain or the ΔP_iC strain were subjected to immunodecoration with antiserum directed against Hsp60 as described previously (22). (B) Import into isolated mitochondria. Rabbit reticulocyte lysates containing the radiolabeled preproteins of the ADP/ATP carrier (AAC) and Fe/S protein were incubated (in BSA buffer) with *S. cerevisiae* mitochondria (5 μg of protein) isolated from the wild-type strain or from the ΔP_iC strain. Lanes 1, 3, 5, and 7 contained 2 mM NADH (to generate a membrane potential, $\Delta\psi$) and lanes 2, 4, 6, and 8 contained valinomycin and oligomycin (to dissipate the $\Delta\psi$). Incubation was for 5 min at 25°C. The mitochondria were reisolated and treated with proteinase K (250 μg/ml). Analysis was performed by SDS-PAGE and fluorography. The asterisk indicates fragments of outer membrane-accumulated precursor of AAC that were generated by added proteinase K. (C) Import into isolated mitochondria. Reticulocyte lysates containing the radiolabeled preprotein Su9-DHFR were incubated with isolated mitochondria in BSA buffer in the presence of a membrane potential for the indicated times as described for panel B. The reisolated mitochondria were treated with proteinase K. Analysis was by SDS-PAGE and laser densitometry of the fluorographs. The amount of Su9-DHFR imported into wild-type mitochondria after 20 min was set to 100% (control). p, i, and m, precursor-, intermediate-, and mature-sized form of a protein, respectively.

(33). The Fe/S protein and Su9-DHFR are proteolytically cleaved during import (removal of the N-terminal targeting sequence), whereas the ADP/ATP carrier contains internal targeting sequences and thus is not cleaved. With ΔP_iC mitochondria, the import of all three preproteins was strongly inhibited (Fig. 1B, lanes 5 and 7, and Fig. 1C).

The possibility that the strong reduction of protein import was due to a disturbance of the overall protein content or stability of ΔP_iC mitochondrial membranes was of concern. The overall protein patterns of ΔP_iC mitochondria (after separation by SDS-PAGE and staining with Coomassie brilliant blue R250) were comparable for ΔP_iC and wild-type mitochondria except that a prominent protein band of 33 kDa was

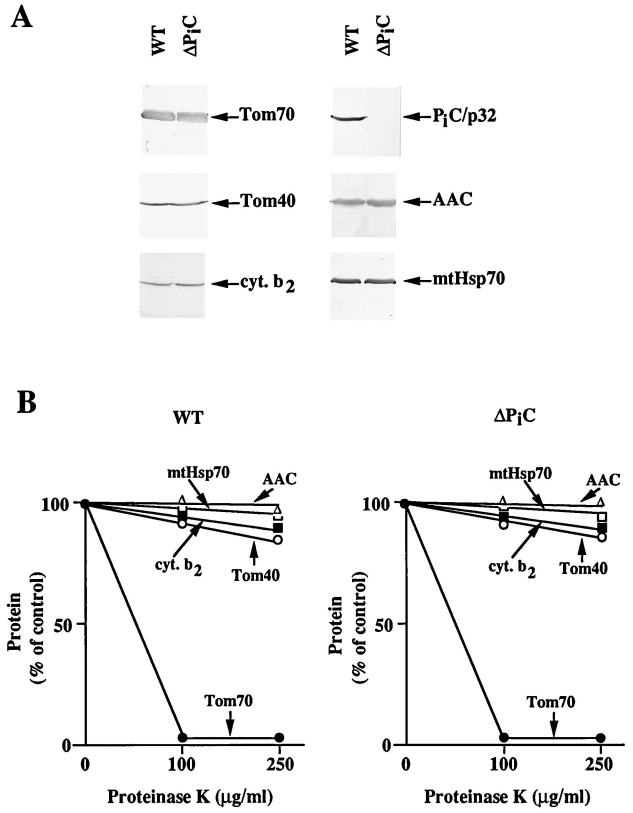

FIG. 2. The stability of ΔP_iC mitochondrial membranes is not decreased. (A) Wild-type (WT) and ΔP_iC mitochondria (100 μg of protein) were separated by SDS-PAGE, transferred to nitrocellulose, and immunodecorated with antibodies directed against the outer membrane proteins Tom70 and Tom40, cytochrome b_2 (cyt. b_2) of the intermembrane space, P_iC/p32, ADP/ATP carrier (AAC) of the inner membrane, and matrix Hsp70 (mtHsp70) (11, 37). (B) Wild-type and ΔP_iC mitochondria (0.5 mg of protein per ml in 250 mM sucrose–10 mM Tris–1 mM EDTA, pH 7.2) were treated with proteinase K for 15 min at 0°C, and then 1 mM phenylmethylsulfonyl fluoride was added. The mitochondria were reisolated and analyzed by immunodecoration as described for panel A and by densitometry. The amount of a protein in the absence of protease treatment was set to 100% (control). Similarly, when the treatment was performed with trypsin, the protease protections of wild-type and ΔP_iC mitochondria were indistinguishable.

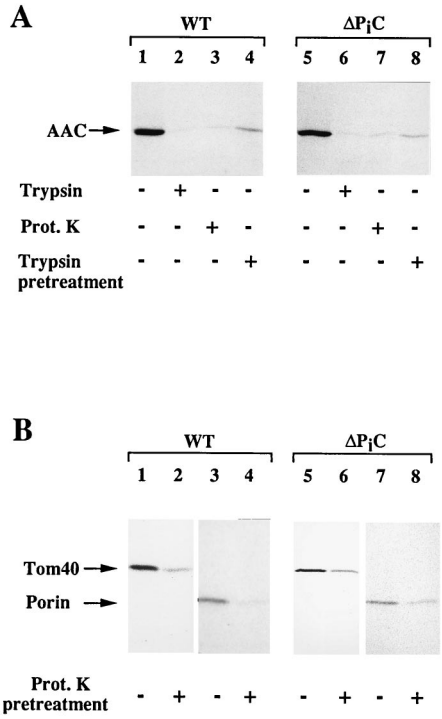

FIG. 3. ΔP_iC mitochondria can bind a preprotein to outer membrane receptor sites and import outer membrane proteins. (A) Binding of the precursor of ADP/ATP carrier to outer membrane receptor sites. Wild-type (WT) and ΔP_iC mitochondria were either pretreated with trypsin (lanes 4 and 8) to remove the surface receptor domains (1, 42) or left untreated. Reticulocyte lysates containing the radiolabeled preprotein of the ADP/ATP carrier (AAC) were depleted of ATP by preincubation with apyrase (5 U/ml) for 15 min at 25°C and then incubated with the mitochondria (pretreated with 5 U of apyrase per ml for 25 min at 0°C) in the presence of 1 μM valinomycin, 20 μM oligomycin, and 8 μM antimycin A for 7 min at 25°C. The mitochondria in lanes 2 and 6 were then treated with trypsin (20 μg/ml), and the mitochondria in lanes 3 and 7 were treated with proteinase K (Prot. K) (20 μg/ml). The mitochondria of all samples were reisolated and analyzed by SDS-PAGE and fluorography. (B) Import of outer membrane proteins. Wild-type and ΔP_iC mitochondria were pretreated with proteinase K (20 μg/ml) (lanes 2, 4, 6, and 8) or left untreated (lanes 1, 3, 5, and 7). Reticulocyte lysates containing the radiolabeled precursors of Tom40 or porin were incubated with the mitochondria for 7 min at 25°C in a final volume of 100 μl. The mitochondria were then treated with 100 μg of trypsin per ml and reisolated.

lacking in the mutant mitochondria (not shown). To directly demonstrate that the band represented P_iC/p32, the proteins were transferred to nitrocellulose and immunodecorated with specific antibodies. Figure 2A demonstrates that P_iC/p32 was completely absent in the mutant mitochondria, whereas marker proteins for the four mitochondrial subcompartments, i.e., outer membrane (the import receptor Tom70 and Tom40 of the general import pore [21, 32, 38]), intermembrane space (cytochrome b_2), inner membrane (ADP/ATP carrier), and matrix (the heat shock protein mtHsp70), were present in comparable amounts in wild-type and mutant mitochondria. In particular, components of the protein import machinery, such as Tom70, Tom40, and matrix Hsp70 (15, 17, 43, 46), were present in wild-type amounts in the mutant mitochondria. Thus the reduced rate of protein import does not disturb the overall protein composition of ΔP_iC mitochondria (the following explanations are conceivable: at the reduced growth rate of ΔP_iC cells, the reduced protein import is still sufficient for biogenesis of mitochondria; or, alternatively, the reduced protein import is rate limiting for mitochondrial and cellular growth such that only mitochondria which show a physiological protein composition are formed). Moreover, the stabilities of the mitochondrial membranes in wild-type and mutant mitochondria were comparable: treatment of the mitochondria with high concentrations of protease (up to 250 μg/ml) followed by analysis of the marker proteins did not reveal a decreased stability of the mutant mitochondria (Fig. 2B) (Tom70 is exposed on the outer membrane surface and is thus degraded by protease). We conclude that the ΔP_iC mitochondrial membranes are of high stability like wild-type mitochondrial membranes.

ΔP_iC mitochondria are not blocked in binding of preproteins to receptor sites and import of outer membrane proteins. We asked which stage of protein import was affected in ΔP_iC mitochondria. The precursor of the ADP/ATP carrier can be bound to outer membrane receptor sites when it is incubated with isolated mitochondria in the absence of a membrane potential ($\Delta\psi$) across the inner membrane and after depletion of ATP (1, 42). ^{35}S-labeled precursor of the ADP/ATP carrier was incubated with isolated wild-type and ΔP_iC mitochondria under these conditions. The bound precursor (Fig. 3A, lanes 1 and 5) was exposed on the outer membrane surface, as it was completely degraded by addition of an external protease (tryp-

sin or proteinase K) (Fig. 3A, lanes 2, 3, 6, and 7). To assess the specificity of the binding reaction, the mitochondria were pretreated with a low concentration of trypsin, which removes the outer membrane receptor sites (1, 42), and then incubated with the precursor protein; lanes 4 and 8 of Fig. 3A demonstrate that the binding to both wild-type and ΔP_iC mitochondria was strongly decreased. Therefore, the binding of the ADP/ATP carrier to the mitochondrial surface observed in lanes 1 and 5 of Fig. 3A represents binding to proteinaceous receptor sites. ΔP_iC mitochondria bound the same amount of preprotein as wild-type mitochondria. We conclude that ΔP_iC mitochondria are not blocked in an initial import stage, i.e., preprotein binding to the outer membrane surface.

We then investigated whether ΔP_iC mitochondria were affected in the subsequent import step, insertion of proteins into the outer membrane. We used the ^{35}S-labeled precursors of two outer membrane proteins, Tom40 and porin. The preproteins were incubated with isolated mitochondria and then treated with protease such that only fully imported proteins were resistant (16, 18, 22). ΔP_iC mitochondria were competent in import of both Tom40 and porin (Fig. 3B, lanes 5 and 7). The specificity of the import reaction was analyzed by using mitochondria that were pretreated with a low concentration of protease to remove the surface receptor sites; under these conditions, the import of Tom40 and porin was strongly inhibited, with both wild-type mitochondria and ΔP_iC mitochondria (Fig. 3B, lanes 2, 4, 6, and 8). We conclude that the removal of outer membrane receptor sites, but not the lack of P_iC, inhibits insertion of preproteins into the outer membrane.

Similarly, the experiments with import of the ADP/ATP carrier shown in Fig. 1B suggest that the ΔP_iC mitochondria were able to insert preproteins into the outer membrane. The asterisk in Fig. 1B indicates fragments of not fully imported precursor of ADP/ATP carrier that were generated by added proteinase K. In wild-type mitochondria, the fragments were observed only when the precursor was accumulated at the general import pore of the outer membrane (incubation with mitochondria in the absence of a membrane potential [Fig. 1B, lane 2]) (1). These fragments were the major forms of the ADP/ATP carrier observed with ΔP_iC mitochondria (Fig. 1B, lanes 5 and 6).

ΔP_iC mitochondria have a decreased membrane potential. Since ΔP_iC mitochondria were not inhibited in preprotein binding to and insertion into the outer membrane, it was possible that the defect in protein import occurred at the level of transport across the inner membrane. The presence of a membrane potential ($\Delta\psi$) across the inner membrane is an obligatory prerequisite for any preprotein transport into or across this membrane (14, 42). We thus assessed the membrane potential of ΔP_iC mitochondria by use of the fluorescent dye $DiSC_3(5)$ (39). The difference between the fluorescence after addition of mitochondria and substrates and that after the subsequent addition of the potassium ionophore valinomycin (in the presence of external potassium, leading to a complete dissipation of $\Delta\psi$) is taken as an assessment of the mitochondrial membrane potential (8, 10). The valinomycin-sensitive fluorescence decrease observed with ΔP_iC mitochondria was only about 10% of that observed with wild-type mitochondria (mean ± standard error of the mean for seven independent experiments, 0.10 ± 0.01) (Fig. 4A), demonstrating that ΔP_iC mitochondria had a strongly decreased membrane potential.

The isolated mitochondria apparently contained a sufficient amount of substrates, since the addition of an external substrate (succinate) did not lead to a further fluorescence decrease (Fig. 4A) as was previously observed with various yeast wild-type and mutant mitochondria (2, 10). However, we found that the addition of phosphate to the buffer was essential for measurement of a valinomycin-sensitive fluorescence decrease with yeast mitochondria (Fig. 4A, upper panel); this is in contrast to results with mammalian mitochondria, where generation of a membrane potential was possible without addition of phosphate (30). Mersalyl, an inhibitor of the phosphate carriers of *S. cerevisiae* and mammals (6, 44), prevented the generation of a membrane potential by yeast mitochondria but not by mammalian mitochondria (data not shown), suggesting that phosphate import across the inner membrane of yeast mitochondria was required for establishing a $\Delta\psi$. The implications of this property of yeast mitochondria will be discussed below.

We studied whether it was possible to enhance the low membrane potential observed with ΔP_iC mitochondria and finally found that the addition of a high concentration of ATP (5 mM) led to a significant increase of the assessed $\Delta\psi$ (Fig. 4B).

Restoration of a membrane potential restores protein import into ΔP_iC mitochondria. If the strong decrease in the membrane potential caused the import defect of ΔP_iC mitochondria, a restoration of $\Delta\psi$ by the addition of ATP at a high concentration should also lead to an increase of protein import. Indeed, the addition of 5 mM ATP strongly increased the import of Su9-DHFR into ΔP_iC mitochondria (Fig. 5A, lane 8 and Fig. 5B). The ratio of import into ΔP_iC mitochondria to that into wild-type mitochondria (mean ± standard error of the mean for seven independent experiments) was 0.10 ± 0.01 without ATP and 0.53 ± 0.04 with 5 mM ATP, and this increase of protein import correlated well with the increase of the inner membrane potential (assessed by fluorescence quenching; ratios of 0.10 ± 0.01 and 0.57 ± 0.05, respectively). The import stimulation by addition of a high ATP concentration was inhibited by addition of oligomycin, which blocks the mitochondrial F_0F_1-ATPase (Fig. 5A, lane 10).

The transport of ATP into mitochondria can be selectively blocked by addition of atractyloside, which inhibits the ADP/ATP carrier of the inner membrane (31). The protein import-restoring effect of ATP on ΔP_iC mitochondria was blocked by addition of atractyloside (Fig. 5C, lane 6), indicating that the ATP must be transported into the mitochondrial matrix to cause the stimulation of protein import.

The restoration of a $\Delta\psi$ also restored the import of the precursor of the ADP/ATP carrier, as evidenced by the protease protection of the full-length ADP/ATP carrier (Fig. 5D, lane 6) and the disappearance of the proteinase K-generated fragment of the ADP/ATP carrier, which is indicative of outer membrane accumulation of the precursor (Fig. 5D, lane 4, asterisk). It was previously shown that the import of the ADP/ATP carrier into the inner membrane of wild-type mitochondria could also efficiently occur when the matrix was depleted of ATP (33, 47). This indicates that the inhibition of import into ΔP_iC mitochondria was not directly caused by a decreased matrix ATP level (but was due to the decreased $\Delta\psi$) and that the import-stimulating effect of addition of ATP occurred via enhancement of the membrane potential, as discussed below.

The restoring conditions do not influence the block in phosphate import of ΔP_iC mitochondria. Two methods were used to measure the mitochondrial import of phosphate: swelling of mitochondria after addition of ammonium phosphate (Fig. 6A) and uptake of [^{32}P]phosphate (Fig. 6B). Both methods demonstrated strong inhibition of phosphate import by ΔP_iC mitochondria. As reported previously (6, 12), the yeast mitochondrial P_iC/p32 was not inhibited by the sulfhydryl reagent *N*-ethylmaleimide but was inhibited by mersalyl (Fig. 6A, wild-type). The rates of uptake of phosphate by wild-type yeast

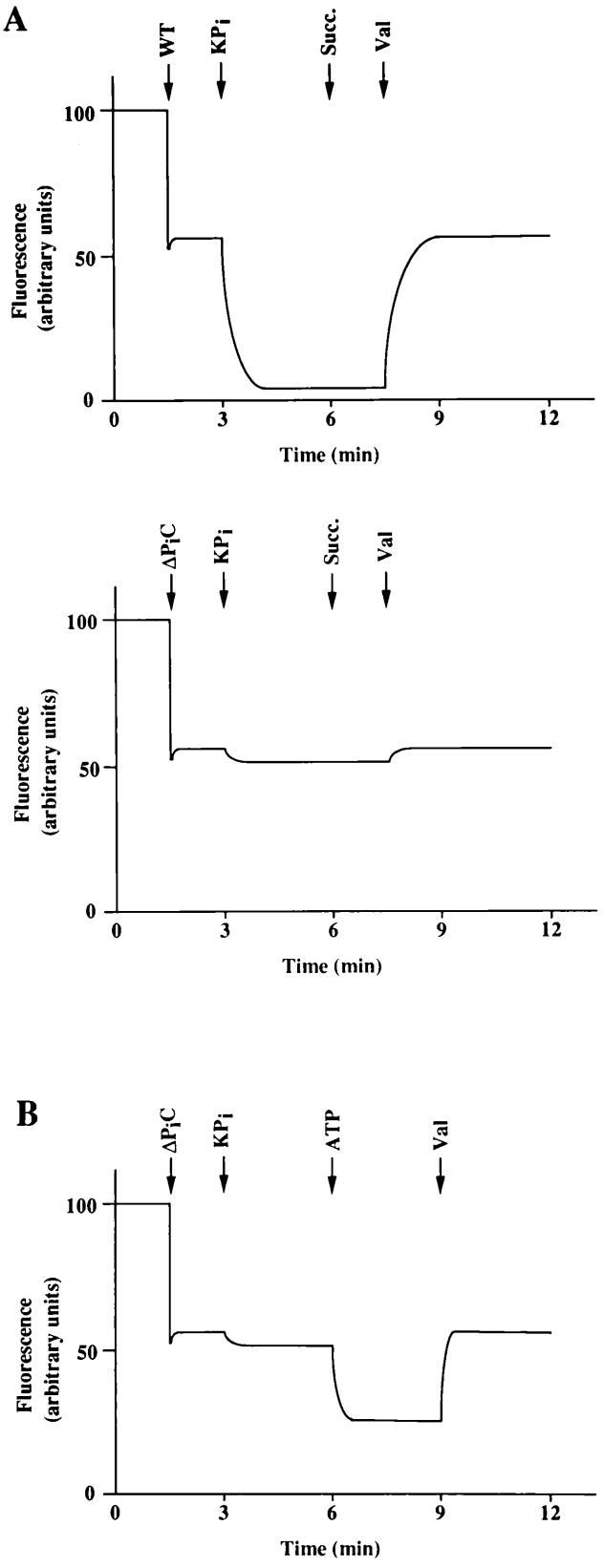

FIG. 4. Assessment of the membrane potential of ΔP_iC mitochondria. (A) Strong decrease of the membrane potential in ΔP_iC mitochondria. Wild-type (WT) and ΔP_iC mitochondria (25 μg of protein) were incubated with the membrane potential-sensitive dye $DiSC_3(5)$, and the fluorescence changes were recorded as described in Materials and Methods. When indicated, 20 mM phosphate, 5 mM succinate (Succ.), or 1 μM valinomycin (Val) was added. (B) Enhancement of the membrane potential of ΔP_iC mitochondria by addition of ATP. ΔP_iC mitochondria (25 μg of protein) were incubated with $DiSC_3(5)$ as described above. When indicated, 20 mM phosphate, 5 mM Mg-ATP, or 1 μM valinomycin was added.

mitochondria (Fig. 6B) were comparable to those found for mammalian mitochondria (4).

Do the conditions that restore a $\Delta\psi$ and protein import (addition of 5 mM ATP) also restore import of phosphate? Figure 6 demonstrates that this was not the case. The addition of ATP did not promote phosphate uptake by ΔP_iC mitochondria. We conclude that restoration of the $\Delta\psi$ and protein import by ΔP_iC mitochondria is not coupled to transport of P_iC across the inner membrane.

DISCUSSION

It has been proposed that the mitochondrial P_iC/p32 is a bifunctional protein, responsible for both protein import and phosphate transport (24, 25). Our investigations with an *S. cerevisiae* mutant lacking P_iC/p32 indeed initially suggested a dual role of P_iC/p32, as the ΔP_iC mitochondria were strongly inhibited in protein import and blocked in phosphate uptake.

The transport of preproteins into the inner membrane or matrix of isolated mitochondria was strongly reduced with ΔP_iC mitochondria compared with wild-type mitochondria. Since the import of preproteins was analyzed by protection against externally added protease, the possibility that the stability of the mutant mitochondrial membranes was impaired was of concern. This possibility could be excluded, since the protease protection of marker proteins for the four mitochondrial subcompartments was indistinguishable for mutant and wild-type mitochondria. The ΔP_iC mitochondria were able to bind preproteins to receptor sites on their surface and to insert proteins into the outer membrane, suggesting that the major block in protein import into ΔP_iC mitochondria occurred at a later stage of the import pathway.

The membrane potential $\Delta\psi$ across the inner membrane of ΔP_iC mitochondria was strongly decreased. Since protein transport into or across the inner membrane requires the presence of a $\Delta\psi$, it was conceivable that the decrease in $\Delta\psi$ was the cause for the protein transport defect. We thus searched for a condition to enhance the $\Delta\psi$ of ΔP_iC mitochondria and found that the addition of a high concentration of ATP led to a significant increase. The increase in the $\Delta\psi$ of ΔP_iC mitochondria indeed resulted in a significant increase in protein import. ATP was needed in the mitochondrial matrix, as the import-restoring effect was blocked by atractyloside, which inactivates the ADP/ATP carrier of the inner membrane (31). The restoring conditions had no effect on the transport of phosphate, which was blocked in ΔP_iC mitochondria both in the absence and in the presence of additional ATP. We conclude that the defect in protein import into ΔP_iC mitochondria is an indirect effect caused by a decrease of the membrane potential and can be relieved after regeneration of a $\Delta\psi$.

Why does a lack of the phosphate carrier lead to a strong reduction of the membrane potential of *S. cerevisiae* mitochondria? ΔP_iC cells showed a high frequency of loss of mitochondrial DNA, causing an inactivation of the respiratory chain; no membrane potential was detectable by use of the fluorescent dye with mitochondria isolated from [*rho*$^-$] cells. However, for this report we used mitochondria isolated from [*rho*$^+$] ΔP_iC cells, i.e., mitochondria with a functional mitochondrial genome, and still observed the strong reduction of $\Delta\psi$. *S. cerevi-*

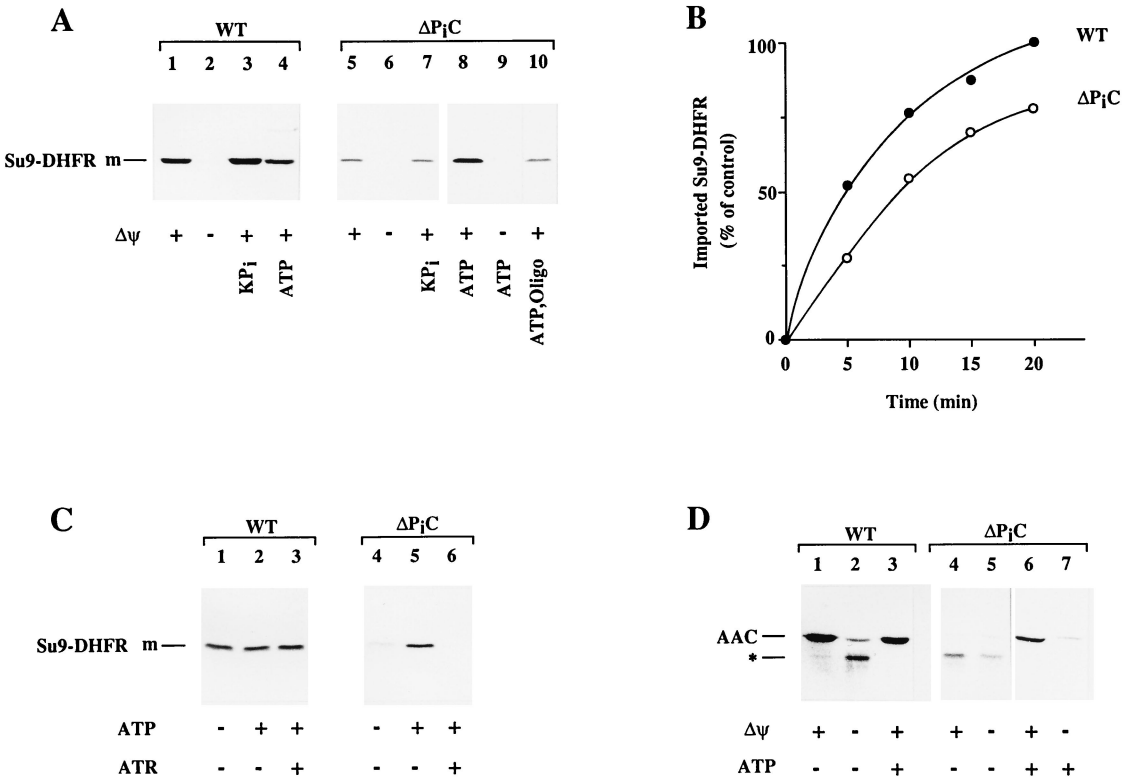

FIG. 5. Restoration of protein import into ΔP_iC mitochondria by addition of a high concentration of ATP. (A) Reticulocyte lysates containing radiolabeled Su9-DHFR were incubated with wild-type (WT) mitochondria (lanes 1 to 4) or ΔP_iC mitochondria (lanes 5 to 10) (5 μg of mitochondrial protein per sample) in BSA buffer at 25°C for 5 min as described in Materials and Methods. Lanes 1, 3 to 5, 7, 8, and 10 contained 2 mM NADH; lanes 3 and 7 contained 10 mM potassium phosphate; lanes 4 and 8 to 10 contained 5 mM Mg-ATP (in addition to the ATP supplied from the reticulocyte lysate); lanes 2, 6, and 9 contained 1 μM valinomycin; and lane 10 contained 40 μM oligomycin (Oligo). The mitochondria were reisolated and treated with proteinase K (250 μg/ml). m, mature-sized form. (B) Su9-DHFR was imported into isolated wild-type and ΔP_iC mitochondria in the presence of a $\Delta\psi$ and added 5 mM Mg-ATP as described for lane 8 of panel A for the indicated times. The fluorographs were analyzed by laser densitometry. The amount of Su9-DHFR imported into wild-type mitochondria after 20 min was set to 100% (control). (C) Reticulocyte lysates containing radiolabeled Su9-DHFR were incubated with isolated mitochondria for 5 min at 25°C. All lanes contained 2 mM NADH; lanes 2, 3, 5, and 6 contained 5 mM Mg-ATP; and lanes 3 and 6 contained 0.5 mM atractyloside (ATR), a specific inhibitor of the ADP/ATP carrier (31). The mitochondria were then treated with proteinase K (250 μg/ml). (D) Reticulocyte lysates containing the radiolabeled precursor of ADP/ATP carrier (AAC) were incubated with isolated mitochondria (5 μg of protein) for 5 min at 25°C. Lanes 1, 3, 4, and 6 contained 2 mM NADH; lanes 3, 6, and 7 contained 5 mM Mg-ATP; and lanes 2, 5, and 7 contained 1 μM valinomycin. The samples were then treated with proteinase K (250 μg/ml) and analyzed by SDS-PAGE and fluorography. The asterisk indicates fragments of outer membrane-accumulated AAC.

siae mitochondria, but not mammalian mitochondria, were reported to possess an uncoupling activity of the inner membrane that is blocked by addition of phosphate (3, 36, 45). Indeed, we found that the addition of phosphate to isolated yeast mitochondria, but not to mammalian mitochondria, was required for detection of a membrane potential by the fluorescence assay (in the in vitro protein import assays, phosphate is supplied by the reticulocyte lysate). Inactivation of P_iC/p32 by mersalyl prevented the phosphate-induced generation of a membrane potential in yeast mitochondria but not in mammalian mitochondria, suggesting that the phosphate had to be present in the matrix of yeast mitochondria to prevent uncoupling. In ΔP_iC mitochondria, the matrix is depleted of phosphate, and thus the uncoupling activity across the inner membrane should be on, leading to a reduction of $\Delta\psi$.

The addition of a high concentration of ATP enhanced the membrane potential and protein import yet did so only when the ATP could be transported via the ADP/ATP carrier across the inner membrane into the matrix. Moreover, the restoring effect of ATP was blocked by inhibition of the F_0F_1-ATPase by oligomycin. This suggests that, in the absence of oligomycin, the F_0F_1-ATPase hydrolyzes the ATP in the matrix, causing a threefold effect: generation of phosphate, which diminishes an uncoupling activity; generation of ADP, which can be exported from the matrix in exchange for import of more ATP (leading to a net transport of negative charge); and use of the energy from ATP hydrolysis to drive the export of protons. When the uncoupling activity is diminished, the latter two effects can support the generation of a $\Delta\psi$ (positive on the outside). All three effects thus enhance the membrane potential, explaining the stimulation of protein import.

The comparison of wild-type yeast mitochondria and ΔP_iC mitochondria demonstrated that P_iC/p32 represents a major band of the total mitochondrial protein pattern. P_iC/p32 is thus far more abundant than known components of the preprotein translocase of the outer and inner mitochondrial membranes (Tom and Tim proteins [32, 40, 41]). Although it cannot be fully excluded that P_iC/p32 is somehow involved in interaction with preproteins (25), it is now clear that P_iC/p32 is essential only for phosphate transport and not for protein targeting and translocation. The strong defects of ΔP_iC mitochondria in protein import are indirectly caused by a reduction of the membrane potential.

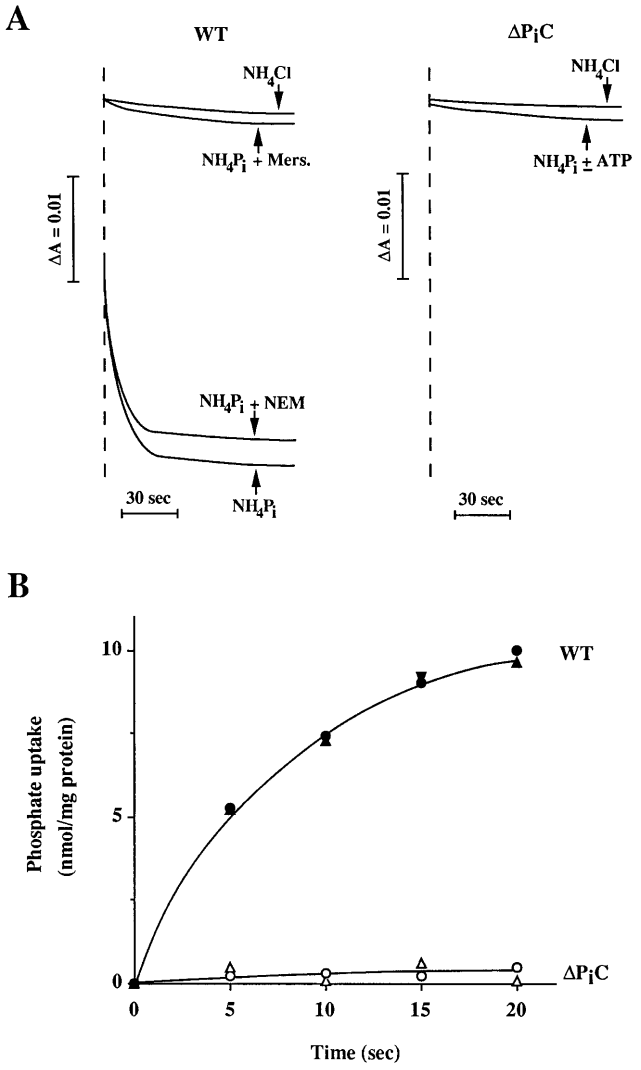

FIG. 6. ΔP_iC mitochondria are blocked in phosphate transport. (A) Turbidity changes corresponding to the swelling of isolated wild-type (WT) and ΔP_iC mitochondria. The incubation buffer contained the indicated ammonium salts at 120 mM, 20 mM Tris, 1 mM EDTA, 5 μM rotenone, and 0.1 mg of mitochondrial protein in a final volume of 1 ml (pH 7.4). The turbidity changes of the mitochondrial suspensions were recorded at 546 nm. When indicated, 1 mM *N*-ethylmaleimide (NEM), 0.5 mM mersalyl (Mers.), or 5 mM ATP was added. (B) Uptake of [^{32}P]phosphate into wild-type and ΔP_iC mitochondria. The time dependence of uptake of phosphate into freshly isolated yeast mitochondria was determined by the inhibitor stop method (29) as described in Materials and Methods. Briefly, 1.35 mM [^{32}P]phosphate was added at time zero to intact mitochondria incubated at 0°C in the presence (triangles) or absence (circles) of 5 mM ATP. The transport reaction was blocked at the times indicated by the addition of 1.5 mM mersalyl. The radioactivity associated with the reisolated mitochondria was counted, and the amount of [^{32}P]phosphate transported into the matrix space was calculated as previously described (29).

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REFERENCES

1. **Alconada, A., F. Gärtner, A. Hönlinger, M. Kübrich, and N. Pfanner.** 1995. Mitochondrial receptor complex from *Neurospora crassa* and *Saccharomyces cerevisiae*. Methods Enzymol. **260:**263–286.
2. **Alconada, A., M. Kübrich, M. Moczko, A. Hönlinger, and N. Pfanner.** 1995. The mitochondrial receptor complex: the small subunit Mom8b/Isp6 supports association of receptors with the general insertion pore and transfer of preproteins. Mol. Cell. Biol. **15:**6196–6205.
3. **Balcavage, W. X., and J. R. Mattoon.** 1968. Properties of *Saccharomyces cerevisiae* mitochondria prepared by a mechanical method. Biochim. Biophys. Acta **153:**521–530.
4. **Coty, W. A., and P. L. Pedersen.** 1974. Phosphate transport in rat liver mitochondria. J. Biol. Chem. **249:**2593–2598.
5. **Daum, G., P. C. Böhni, and G. Schatz.** 1982. Import of proteins into mitochondria: cytochrome b_2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. J. Biol. Chem. **257:** 13028–13033.
6. **de Chateaubodeau, G., M. Guérin, and B. Guérin.** 1974. Studies on anionic transport in yeast mitochondria and promitochondria. Swelling in ammonium phosphate, glutamate, succinate and fumarate solutions. FEBS Lett. **46:**184–187.
7. **Dietmeier, K., V. Zara, A. Palmisano, F. Palmieri, W. Voos, J. Schlossmann, M. Moczko, G. Kispal, and N. Pfanner.** 1993. Targeting and translocation of the phosphate carrier/p32 to the inner membrane of yeast mitochondria. J. Biol. Chem. **268:**25958–25964.
8. **Eilers, M., W. Oppliger, and G. Schatz.** 1987. Both ATP and an energized inner membrane are required to import a purified precursor protein into mitochondria. EMBO J. **6:**1073–1077.
9. **Ferreira, G. C., R. D. Pratt, and P. L. Pedersen.** 1990. Mitochondrial proton/phosphate transporter. An antibody directed against the COOH terminus and proteolytic cleavage experiments provides new insights about its membrane topology. J. Biol. Chem. **265:**21202–21206.
10. **Gärtner, F., W. Voos, A. Querol, B. Miller, E. A. Craig, M. G. Cumsky, and N. Pfanner.** 1995. Mitochondrial import of subunit Va of cytochrome *c* oxidase characterized with yeast mutants: independence from receptors, but requirement for matrix hsp70 translocase function. J. Biol. Chem. **270:**3788–3795.
11. **Glick, B. S., A. Brandt, K. Cunningham, S. Müller, R. L. Hallberg, and G. Schatz.** 1992. Cytochromes c_1 and b_2 are sorted to the intermembrane space of yeast mitochondria by a stop-transfer mechanism. Cell **69:**809–822.
12. **Guérin, B., C. Bukusoglu, F. Rakotomanana, and H. Wohlrab.** 1990. Mitochondrial phosphate transport. N-ethylmaleimide insensitivity correlates with absence of beef heart-like Cys^{42} from the *Saccharomyces cerevisiae* phosphate transport protein. J. Biol. Chem. **265:**19736–19741.
13. **Hartl, F.-U., J. Ostermann, B. Guiard, and W. Neupert.** 1987. Successive translocation into and out of the mitochondrial matrix: targeting of proteins to the intermembrane space by a bipartite signal peptide. Cell **51:**1027–1037.
14. **Hartl, F.-U., N. Pfanner, D. W. Nicholson, and W. Neupert.** 1989. Mitochondrial protein import. Biochim. Biophys. Acta **988:**1–45.
15. **Hines, V., A. Brandt, G. Griffiths, H. Horstmann, H. Brütsch, and G. Schatz.** 1990. Protein import into yeast mitochondria is accelerated by the outer membrane protein MAS70. EMBO J. **9:**3191–3200.
16. **Hönlinger, A., U. Bömer, A. Alconada, C. Eckerskorn, F. Lottspeich, K. Dietmeier, and N. Pfanner.** 1996. Tom7 modulates the dynamics of the mitochondrial outer membrane translocase and plays a pathway-related role in protein import. EMBO J. **15:**2125–2137.
17. **Kang, P. J., J. Ostermann, J. Shilling, W. Neupert, E. A. Craig, and N. Pfanner.** 1990. Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. Nature (London) **348:**137–143.
18. **Keil, P., A. Weinzierl, M. Kiebler, K. Dietmeier, T. Söllner, and N. Pfanner.** 1993. Biogenesis of the mitochondrial receptor complex: two receptors are required for binding of MOM38 to the outer membrane surface. J. Biol. Chem. **268:**19177–19180.
19. **Laloraya, S., P. J. T. Dekker, W. Voos, E. A. Craig, and N. Pfanner.** 1995. Mitochondrial GrpE modulates the function of matrix Hsp70 in translocation and maturation of preproteins. Mol. Cell. Biol. **15:**7098–7105.
20. **Leighton, J., and G. Schatz.** 1995. An ABC transporter in the mitochondrial inner membrane is required for normal growth of yeast. EMBO J. **14:**188–195.
21. **Lill, R., and W. Neupert.** 1996. Mechanisms of protein import across the mitochondrial outer membrane. Trends Cell Biol. **6:**56–61.
22. **Moczko, M., B. Ehmann, F. Gärtner, A. Hönlinger, E. Schäfer, and N. Pfanner.** 1994. Deletion of the receptor MOM19 strongly impairs import of cleavable preproteins into *Saccharomyces cerevisiae* mitochondria. J. Biol. Chem. **269:**9045–9051.
23. **Moczko, M., B. Schönfisch, W. Voos, N. Pfanner, and J. Rassow.** 1995. The mitochondrial ClpB homolog Hsp78 cooperates with matrix Hsp70 in maintenance of mitochondrial function. J. Mol. Biol. **254:**538–543.
24. **Murakami, H., G. Blobel, and D. Pain.** 1990. Isolation and characterization of the gene for a yeast mitochondrial import receptor. Nature (London) **347:**488–491.
25. **Murakami, H., G. Blobel, and D. Pain.** 1993. Signal sequence region of mitochondrial precursor proteins binds to mitochondrial import receptor. Proc. Natl. Acad. Sci. USA **90:**3358–3362.
26. **Myers, A. M., L. K. Pape, and A. Tzagoloff.** 1985. Mitochondrial protein synthesis is required for maintenance of intact mitochondrial genomes in *Saccharomyces cerevisiae*. EMBO J. **4:**2087–2092.
27. **Pain, D., H. Murakami, and G. Blobel.** 1990. Identification of a receptor for

protein import into mitochondria. Nature (London) **347:**444–449.
28. **Palmieri, F., and M. Klingenberg.** 1967. Inhibition of respiration under the control of azide uptake by mitochondria. Eur. J. Biochem. **1:**439–446.
29. **Palmieri, F., and M. Klingenberg.** 1979. Direct methods for measuring metabolite transport and distribution in mitochondria. Methods Enzymol. **56:**279–301.
30. **Palmieri, F., I. Stipani, V. Iacobazzi, and E. Quagliariello.** 1979. Cysteine-sulfinate transport as an example of electrogenic exchange in mitochondria, p. 335–344. *In* E. Quagliariello et al. (ed.), Function and molecular aspects of biomembrane transport. Elsevier, Amsterdam.
31. **Pfaff, E., M. Klingenberg, and H. W. Heldt.** 1965. Unspecific permeation and specific exchange of adenine nucleotides in liver mitochondria. Biochim. Biophys. Acta **104:**312–315.
32. **Pfanner, N., M. G. Douglas, T. Endo, N. J. Hoogenraad, R. E. Jensen, M. Meijer, W. Neupert, G. Schatz, U. K. Schmitz, and G. C. Shore.** 1996. Uniform nomenclature for the protein transport machinery of the mitochondrial membranes. Trends Biochem. Sci. **21:**51–52.
33. **Pfanner, N., M. Tropschug, and W. Neupert.** 1987. Mitochondrial protein import: nucleoside triphosphates are involved in conferring import-competence to precursors. Cell **49:**815–823.
34. **Phelps, A., C. T. Schobert, and H. Wohlrab.** 1991. Cloning and characterization of the mitochondrial phosphate transport protein gene from the yeast *Saccharomyces cerevisiae*. Biochemistry **30:**248–252.
35. **Phelps, A., and H. Wohlrab.** 1991. Mitochondrial phosphate transport. The *Saccharomyces cerevisiae* (threonine 43 to cysteine) mutant protein explicitly identifies transport with genomic sequence. J. Biol. Chem. **266:**19882–19885.
36. **Prieto, S., F. Bouillaud, and E. Rial.** 1995. The mechanism for the ATP-induced uncoupling of respiration in mitochondria of the yeast *Saccharomyces cerevisiae*. Biochem. J. **307:**657–661.
37. **Rassow, J., and N. Pfanner.** 1991. Mitochondrial preproteins en route from the outer membrane to the inner membrane are exposed to the intermembrane space. FEBS Lett. **293:**85–88.
38. **Schatz, G., and B. Dobberstein.** 1996. Common principles of protein translocation across membranes. Science **271:**1519–1526.
39. **Sims, P. J., A. S. Waggoner, C.-H. Wang, and J. F. Hoffmann.** 1974. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. Biochemistry **13:**3315–3330.
40. **Söllner, T., G. Griffiths, R. Pfaller, N. Pfanner, and W. Neupert.** 1989. MOM19, an import receptor for mitochondrial precursor proteins. Cell **59:**1061–1070.
41. **Söllner, T., R. Pfaller, G. Griffiths, N. Pfanner, and W. Neupert.** 1990. A mitochondrial import receptor for the ADP/ATP carrier. Cell **62:**107–115.
42. **Söllner, T., J. Rassow, and N. Pfanner.** 1991. Analysis of mitochondrial protein import using translocation intermediates and specific antibodies. Methods Cell Biol. **34:**345–358.
43. **Steger, H. F., T. Söllner, M. Kiebler, K. A. Dietmeier, R. Pfaller, K. S. Trülzsch, M. Tropschug, W. Neupert, and N. Pfanner.** 1990. Import of ADP/ATP carrier into mitochondria: two receptors act in parallel. J. Cell Biol. **111:**2353–2363.
44. **Tyler, D. D.** 1969. Evidence of a phosphate-transporter system in the inner membrane of isolated mitochondria. Biochem. J. **111:**665–678.
45. **Velours, J., M. Rigoulet, and B. Guérin.** 1977. Protection of yeast mitochondrial structure by phosphate and other H^+-donating anions. FEBS Lett. **81:**18–22.
46. **Vestweber, D., J. Brunner, A. Baker, and G. Schatz.** 1989. A 42K outer-membrane protein is a component of the yeast mitochondrial protein import site. Nature (London) **341:**205–209.
47. **Wachter, C., G. Schatz, and B. S. Glick.** 1992. Role of ATP in the intramitochondrial sorting of cytochrome c_1 and the adenine nucleotide translocator. EMBO J. **11:**4787–4794.
48. **Wohlrab, H., and C. Briggs.** 1994. Yeast mitochondrial phosphate transport protein expressed in *Escherichia coli*. Site-directed mutations at threonine-43 and at a similar location in the second tandem repeat (isoleucine-141). Biochemistry **33:**9371–9375.
49. **Zara, V., F. Palmieri, K. Mahlke, and N. Pfanner.** 1992. The cleavable presequence is not essential for import and assembly of the phosphate carrier of mammalian mitochondria, but enhances the specificity and efficiency of import. J. Biol. Chem. **267:**12077–12081.
50. **Zara, V., J. Rassow, E. Wachter, M. Tropschug, F. Palmieri, W. Neupert, and N. Pfanner.** 1991. Biogenesis of the mitochondrial phosphate carrier. Eur. J. Biochem. **198:**405–410.