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Role of ATP in the intramitochondrial sorting of cytochrome c_1 and the adenine nucleotide translocator

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Import of precursor proteins across the mitochondrial inner membrane requires ATP in the matrix. However, some precursors can still cross the outer membrane in ATP-depleted mitochondria. Here we show that the adenine nucleotide translocator is imported normally into the inner membrane after the matrix has been depleted of ATP. This result supports the earlier suggestion that the translocator inserts into the inner membrane without passing through the matrix. Depletion of matrix ATP also has no detectable effect on the import and maturation of cytochrome c_1, which is targeted to the intermembrane space. It thus seems probable that cytochrome c_1 does not completely cross the inner membrane during its import pathway.

Key words: conservative sorting/intermembrane space/mitochondria/protein import/stop-transfer

Introduction

Proteins that are imported into mitochondria must be sorted to the four organellar compartments: outer membrane, inner membrane, intermembrane space and matrix. Targeting information for these compartments is contained within the precursor proteins, often in the form of N-terminal presequences (Schatz, 1987). The structure of mitochondrial targeting signals and the biochemistry of the import process have been extensively studied (reviewed in Schatz, 1987; Pfanner and Neupert, 1990; Glick and Schatz, 1991).

ATP is required for the import of precursor proteins into the mitochondrial matrix (Pfanner and Neupert, 1986; Chen and Douglas, 1987; Eilers *et al.*, 1987; Beasley *et al.*, 1992). It appears that ATP is utilized outside the mitochondria to modulate the interaction of precursors with cytosolic 'antifolding proteins' (Chen and Douglas, 1987; Pfanner *et al.*, 1987, 1988), including chaperone proteins of the hsp70 class (Deshaies *et al.*, 1988; Murakami *et al.*, 1988; Smith and Yaffe, 1991; Gething and Sambrook, 1992). Some precursors can bypass this external ATP-dependent step (Hwang and Schatz, 1989; Miller and Cumsky, 1991). ATP also functions in the matrix, where it is needed for the translocation of proteins across the mitochondrial inner membrane (Hwang and Schatz, 1989; Hwang *et al.*, 1991; C.Wachter and B.S.Glick, in preparation). This requirement for matrix ATP may reflect the action of mitochondrial hsp70 (mhsp70; Craig *et al.*, 1989; Leustek *et al.*, 1989), which has been implicated in the import of a number of precursors (Kang *et al.*, 1990; Ostermann *et al.*, 1990; Scherer *et al.*, 1990; Manning-Krieg *et al.*, 1991).

When ATP levels are depleted inside the mitochondria, precursors can be translocated across the outer membrane, but they are unable to completely cross the inner membrane (Hwang *et al.*, 1991; Rassow and Pfanner, 1991; C.Wachter and B.S.Glick, in preparation). Thus if a protein passes through the matrix as part of its import pathway, depletion of intramitochondrial ATP will disrupt this pathway. Conversely, if a protein is imported normally into ATP-depleted mitochondria, then it does not pass through the matrix. A potential example of this latter type of protein is the adenine nucleotide translocator, which is thought to assemble into the inner membrane from the external side (Pfanner and Neupert, 1987; Hartl and Neupert, 1990; Liu *et al.*, 1990; Mahlke *et al.*, 1990). Indeed, we report here that import and assembly of the translocator can take place in ATP-depleted mitochondria.

We have also investigated the ATP requirements for import and processing of cytochrome c_1. This protein is synthesized with a bipartite presequence. The N-terminal portion of the presequence resembles a matrix-targeting signal and the C-terminal portion contains targeting information for the intermembrane space (van Loon *et al.*, 1986, 1987). Our previous work suggested that this C-terminal domain functions as a 'stop-transfer' signal for the inner membrane, so that cytochrome c_1 reaches the intermembrane space by crossing only the outer membrane (van Loon and Schatz, 1987; Glick *et al.*, 1992). An alternative view is that cytochrome c_1 follows a 'conservative sorting' pathway, in which the precursor is imported into the matrix and then re-exported across the inner membrane (Hartl *et al.*, 1987; Nicholson *et al.*, 1989; Hartl and Neupert, 1990; Stuart *et al.*, 1990). Here we show that import and maturation of cytochrome c_1 occur normally when the mitochondria are depleted of ATP, providing further evidence that cytochrome c_1 does not cross the inner membrane on its way to the intermembrane space.

Results

Import and assembly of the adenine nucleotide translocator are not affected by depleting the matrix of ATP

ATP is needed for import of the adenine nucleotide translocator (Pfanner *et al.*, 1987). To determine where ATP is utilized, we tested whether the translocator could be imported after ATP levels had been depleted either in the matrix (Figure 1A), outside the mitochondria (Figure 1C) or in both locations (Figure 1B). In each case the relevant control incubation contained ATP both inside and outside the mitochondria. The reactions were for 10 min at 12°C, which was in the linear range for import into fully energized mitochondria (not shown). Since the translocator does not

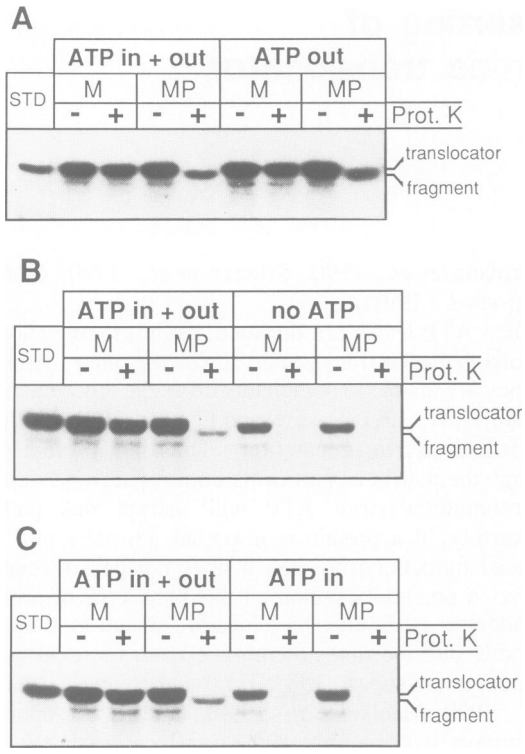

Fig. 1. Import of the adenine nucleotide translocator requires external ATP, but is unaffected by depleting matrix ATP. ATP levels were depleted either (**A**) in the matrix, (**C**) outside the inner membrane or (**B**) in both locations. Radiolabelled precursor of the adenine nucleotide translocator was then incubated with the mitochondria for 10 min at 12°C. Control reactions (ATP in + out) contained ATP on both sides of the inner membrane. Import was terminated by adding FCCP to 25 μM. Where indicated, the mitochondria (M) were treated with proteinase K (Prot. K) or converted to mitoplasts (MP) in the presence or absence of proteinase K. Samples were subjected to SDS–PAGE and fluorography. STD represents 12% of the amount of radiolabelled precursor added in each reaction. The bands corresponding to the full-length translocator and its protease-resistant fragment are indicated.

undergo proteolytic processing, import was measured as the amount of the full-length species protected from proteinase K in intact mitochondria. Import was blocked by depleting ATP outside the mitochondria (Figure 1B and C), but was unaffected by depleting matrix ATP (Figure 1A).

If the imported translocator had assembled into the inner membrane, it should have acquired the properties of the endogenous, pre-existing translocator. The endogenous form was largely protease-resistant (Rassow and Pfanner, 1991): it was digested only to a slightly smaller fragment by proteinase K when the outer membrane was selectively disrupted to generate mitoplasts (Figure 2D), or when the mitochondria were solubilized with Triton X-100. In contrast, most of the translocator that had been imported at 12°C was digested completely by proteinase K in mitoplasts (Figure 1A–C), indicating that assembly had not occurred at this low temperature. However, after import at 25°C, the protease-resistant form was generated (Figure 2A); this form was probably fully assembled as it was unable to bind to hydroxylapatite (not shown; Schleyer and Neupert, 1984). Assembly of the translocator was not affected by depleting matrix ATP (Figure 2A–C).

To confirm that intramitochondrial ATP had been efficiently depleted (Figure 2B), we co-imported the translocator with pSu9(1–69)–DHFR, an artificial matrix-targeted precursor (Pfanner *et al.*, 1987). The presequence of this protein is cleaved in two steps, both of them catalysed by the soluble matrix protease (Schmidt *et al.*, 1984). In fully energized mitochondria, pSu9(1–69)–DHFR was processed to the mature form (m; Figure 2A); this species was protected from protease in mitoplasts and therefore was located in the matrix. In ATP-depleted mitochondria, only trace amounts of pSu9(1–69)–DHFR were transported entirely across the inner membrane and processed to the mature form (Figure 2B). Thus import and assembly of the translocator occurred at normal rates under conditions where ATP-driven import into the matrix was strongly inhibited.

Cytochrome c_1 is imported and processed correctly after depletion of matrix ATP

After import of cytochrome c_1 into mitochondria, two forms were seen (Figure 3D): the intermediate, which had been cleaved only by the matrix protease, and the mature form, in which the second part of the presequence had been removed by an unidentified protease at the outer face of the inner membrane (Ohashi *et al.*, 1982; van Loon *et al.*, 1986). Import required ATP outside the mitochondria (Figure 3D; Hartl *et al.*, 1988). In contrast, when ATP was selectively depleted in the matrix, the import rate of cytochrome c_1 was only slightly diminished (Figure 3A and C). This reduction can be attributed to the oligomycin/efrapeptin mixture, which was present only in the 'ATP out' reactions, and which non-specifically inhibited cytochrome c_1 import by ~25% under these conditions (not shown). Imported cytochrome c_1 was digested by protease in mitoplasts and therefore faced the intermembrane space, both in fully energized and in ATP-depleted mitochondria (Figure 3B). As a control for the effectiveness of the ATP depletion, the hsp60 precursor was co-imported with cytochrome c_1. Hsp60 was translocated into the matrix of the fully energized mitochondria (Figure 3A and B). After ATP depletion, hsp60 was still imported across the outer membrane and processed to the mature form (Figure 3A), but the imported molecules were accessible to protease in mitoplasts (Figure 3B; Hwang *et al.*, 1991). Thus complete translocation of hsp60 across the inner membrane was prevented by depleting matrix ATP, whereas import of cytochrome c_1 to the intermembrane space was essentially unaffected.

The second processing step of cytochrome c_1 requires prior attachment of heme to the apoprotein (Figure 4C; Ohashi *et al.*, 1982) by the enzyme cytochrome c_1 heme lyase (Nicholson *et al.*, 1989). This series of reactions occurred at similar rates in fully energized and in ATP-depleted mitochondria (Figures 3A and C, and 4A). Cytochrome c_1 undergoes an additional reaction: it becomes tightly associated with the inner membrane, probably because a hydrophobic sequence near the protein's C-terminus inserts into the lipid bilayer (Hase *et al.*, 1987). To determine whether membrane insertion of newly imported cytochrome c_1 had occurred, we extracted the mitochondria with 0.1 M Na_2CO_3 (pH 11.5), a treatment that should disrupt protein–protein interactions (Fujiki *et al.*, 1982), including the interaction of the presequence with the inner membrane translocation machinery (Glick *et al.*, 1992). The imported intermediate and mature forms of cytochrome c_1 were recovered in the membrane pellet following Na_2CO_3

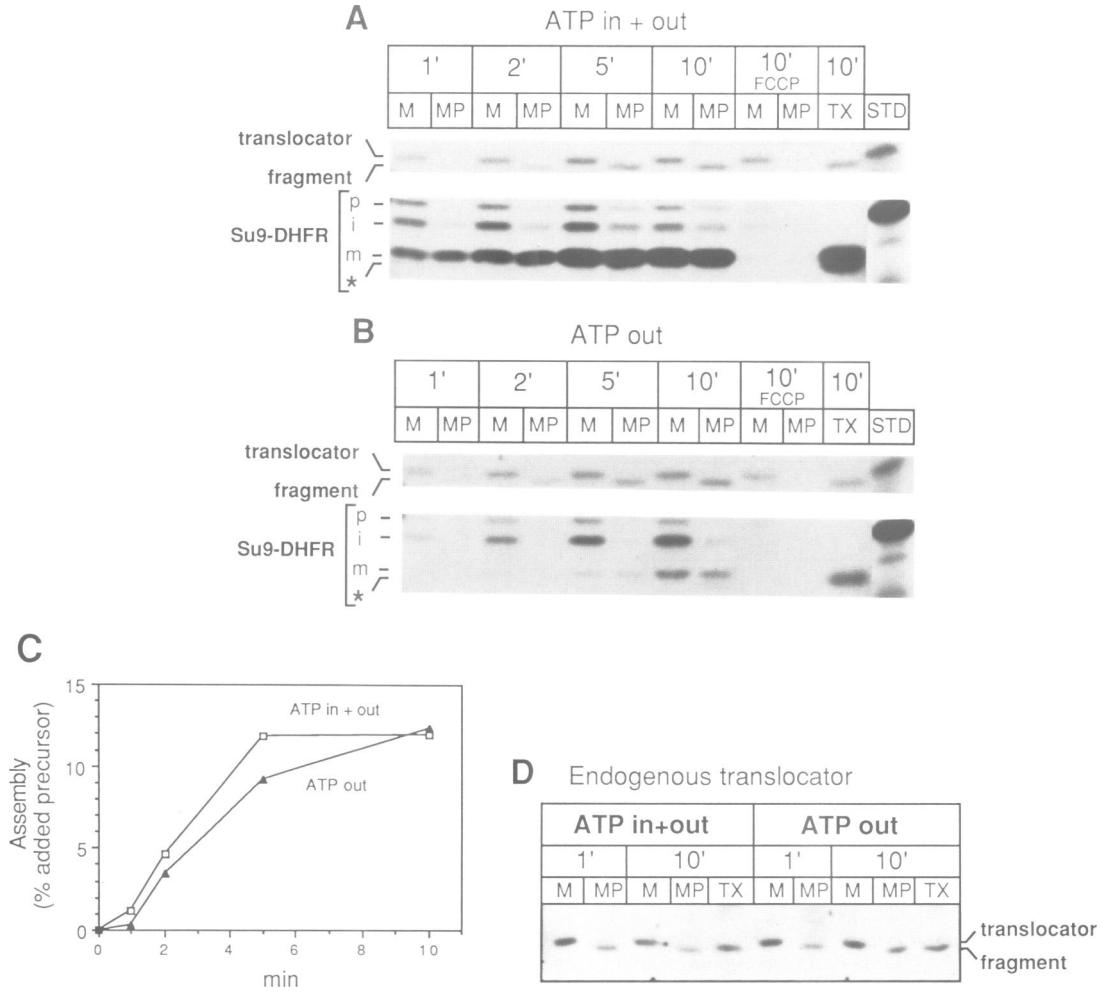

Fig. 2. Import and assembly of the adenine nucleotide translocator follow similar kinetics in fully energized and in ATP-depleted mitochondria. Mitochondria were incubated with a mixture of the pSu9(1−69)−DHFR and adenine nucleotide translocator precursors. Conditions were as described in Materials and methods under 'ATP out', except that the concentrations of CAT and ATP were 2.5 μM and 0.5 mM, respectively. After 1, 2, 5 and 10 min at 25°C, aliquots were removed, adjusted to 25 μM FCCP and transferred to ice. To confirm that assembly required an electrochemical potential across the inner membrane, FCCP was added to a separate aliquot before incubation for 10 min at 25°C. One sample from each aliquot was treated with proteinase K (M), and a second sample was converted to mitoplasts in the presence of proteinase K (MP). With the aliquots incubated for 10 min in the absence of FCCP, a third sample was adjusted to 1% Triton X-100 and treated with proteinase K (TX). Samples were subjected to SDS−PAGE and fluorography. STD: 40% of the amount of precursor mixture initially present in each sample. The positions of the full-length translocator and its protease-resistant fragment are indicated. p, i and m: precursor, intermediate and mature forms of pSu9(1−69)−DHFR. Some folded precursor molecules of pSu9(1−69)−DHFR remained bound to the mitochondrial surface (not shown) and were cleaved by proteinase K to a core fragment of DHFR (*) in the presence of Triton X-100. After complete import into the matrix and ATP-dependent refolding, the mature form of pSu9(1−69)−DHFR was largely resistant to proteinase K digestion (TX in panel A; Ostermann *et al.*, 1989), being converted only slowly to this core fragment (not shown). (**A**) ATP was present on both sides of the inner membrane. (**B**) Matrix ATP was selectively depleted. (**C**) Quantification of the amounts of assembled translocator (MP lanes) from panels A and B. (**D**) A portion of each sample from the 1 and 10 min incubations was used for immunoblotting with an anti-translocator antibody. This antibody had a lower affinity for the translocator fragment than for the full-length protein (not shown), so the signal was somewhat weaker for the fragment.

treatment (Figure 4A). Unlike endogenous cytochrome c_1, which was presumably integrated into the cytochrome bc_1 complex, the newly imported molecules were sensitive to protease in mitoplasts, indicating that their association with membranes was not due to assembly into such a complex. A truncated version of cytochrome c_1, which lacks the C-terminal anchor sequence (Hase *et al.*, 1987), was completely soluble in Na_2CO_3 after import (Figure 4B), further suggesting that the Na_2CO_3-inextractability of the full-length protein reflects membrane insertion of this anchor sequence. Interestingly, membrane insertion of full-length cytochrome c_1 did not require addition of heme to the apoprotein (Figure 4C). As judged by this Na_2CO_3 extraction assay, quantitative membrane insertion of cytochrome c_1 occurred in ATP-depleted mitochondria (Figure 4A). All of our results therefore indicate that the *in vitro* import pathway of cytochrome c_1 was not altered by depleting matrix ATP.

Complete processing of the cytochrome c_1 presequence in ATP-depleted mitochondria apparently reflects import by the normal pathway

$pc_1(1-64)-$DHFR is a fusion protein that consists of the cytochrome c_1 presequence joined to dihydrofolate reductase (van Loon *et al.*, 1986). Like authentic cytochrome c_1, $pc_1(1-64)-$DHFR is imported to the intermembrane space and processed to the mature form in ATP-depleted mitochondria as efficiently as in fully energized mitochondria

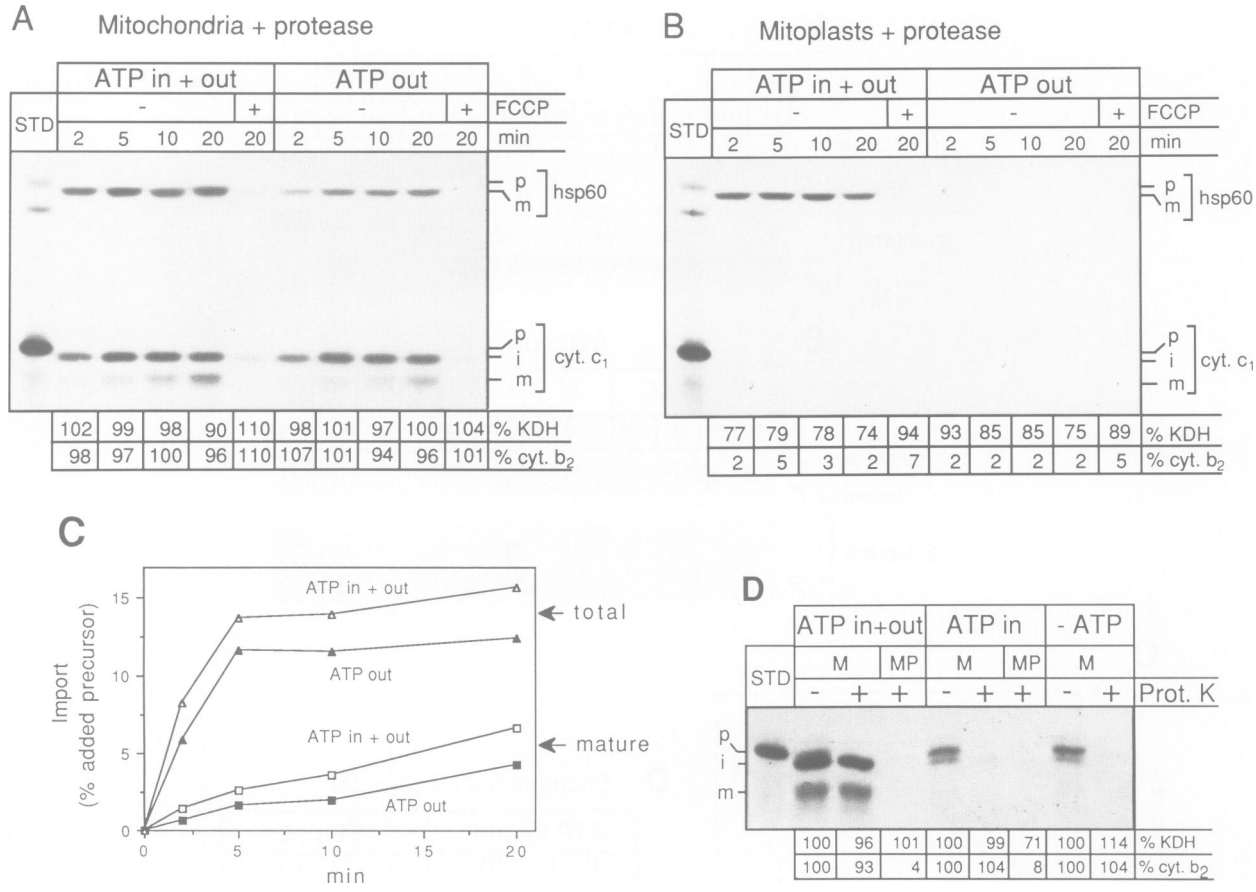

Fig. 3. Import and maturation of cytochrome c_1 are not affected by depleting matrix ATP, but do require external ATP. p and m, precursor and mature forms of hsp60 and cytochrome c_1; i, intermediate form of cytochrome c_1. (**A**–**C**): conditions were as described in Materials and methods under 'ATP out', except that CAT was present at 5 μg/ml, hemin at 1 μM and FMN at 10 μM (Nicholson *et al.*, 1989). A mixture of the cytochrome c_1 and hsp60 precursors (STD, 25% precursor standard) was incubated with mitochondria at 18°C; aliquots were removed after 2, 5, 10 and 20 min and import was stopped as in Figure 2. An additional aliquot contained 25 μM FCCP during a 20 min incubation. One sample from each aliquot was treated with proteinase K (A); a second sample was converted to mitoplasts in the presence of proteinase K (B). These samples were then subjected to SDS–PAGE and fluorography. A third sample from each aliquot was used for immunoblotting to measure the amounts of α-ketoglutarate dehydrogenase (KDH) and cytochrome b_2 (cyt. b_2); these numbers are listed underneath the fluorogram. The 100% level for each marker protein was defined as the average of the values determined for the five samples of protease-treated mitochondria. (C) shows the quantification of the amounts of mature and total imported (intermediate plus mature) cytochrome c_1 from part A. (**D**) Import of cytochrome c_1 was for 25 min at 18°C, with further manipulations as in Figure 1, followed by SDS–PAGE and fluorography. ATP depletion procedures are given in Materials and methods; the control with fully energized mitochondria was performed as described under 'ATP in'. M, MP and Prot. K, as in Figure 1. STD, 15% precursor standard. A portion of each sample was used for immunoblotting as in A and B; the 100% level for each marker protein was defined as the value obtained for the non-protease-treated mitochondria.

(Figure 5A; Glick *et al.*, 1992). We have previously suggested that translocation of $pc_1(1-64)$–DHFR through the inner membrane is arrested by a stop–transfer signal, whether or not ATP is present in the matrix; thus the intermediate form is always correctly positioned for the second cleavage reaction (Glick *et al.*, 1992). However, it could also be argued that $pc_1(1-64)$–DHFR normally follows an import–re-export pathway and that depletion of matrix ATP fortuitously arrests import at a stage where the presequence can undergo the second cleavage step.

To distinguish between these possibilities, we examined the import of the mutant fusion protein $pc_1(1-64,R44)$–DHFR, in which Ala44 of the presequence had been changed to arginine. This substitution abolished the intermembrane space targeting signal and redirected the protein to the matrix (B.S.Glick and K.Cunningham, in preparation). According to the stop–transfer model, $pc_1(1-64,R44)$–DHFR lacks a stop–transfer signal, so it will be arrested in ATP-depleted mitochondria by a different mechanism than $pc_1(1-64)$–DHFR, and thus probably will not be correctly positioned for processing to the mature form. The conservative sorting model predicts that import of both fusion proteins will be arrested by the same mechanism if the matrix is depleted of ATP, so the two proteins should be processed with similar efficiencies.

In fully energized mitochondria, $pc_1(1-64,R44)$–DHFR was imported into the matrix, where it could be cleaved only to the intermediate-sized form (Figure 5A). In ATP-depleted mitochondria, the imported $pc_1(1-64,R44)$–DHFR molecules still were not processed to the mature form, even though they were protease-accessible in mitoplasts and therefore had been arrested during translocation through the inner membrane.

To confirm that the second cleavage site in $pc_1(1-64,R44)$–DHFR was still functional, we took advantage of an unexpected finding: when $pc_1(1-64,R44)$–DHFR was incubated with mitoplasts (see Materials and

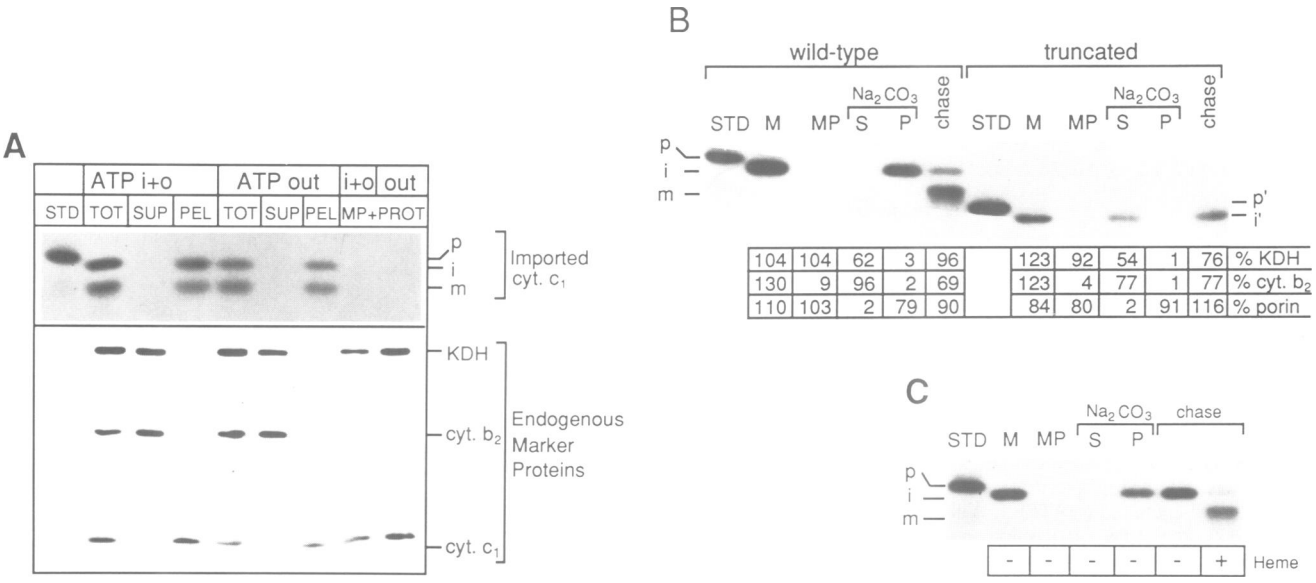

Fig. 4. Cytochrome c_1 is Na_2CO_3-inextractable after import into fully energized or ATP-depleted mitochondria. p, i and m, see Figure 3. (**A**) Cytochrome c_1 was imported for 20 min at 18°C as in Figure 3. The mitochondria were then treated with 50 μg/ml trypsin for 20 min on ice. Soybean trypsin inhibitor was added to 1 mg/ml and the mitochondria were divided into three equal aliquots. The first aliquot was defined as the total (TOT). The second aliquot was treated with Na_2CO_3 and then separated into supernatant (SUP) and membrane pellet (PEL) fractions. The third aliquot was converted to mitoplasts in the presence of proteinase K (MP + PROT). A portion of each sample was taken for SDS–PAGE and fluorography (STD, 15% precursor standard). A second portion was used for immunoblotting with antibodies specific for α-ketoglutarate dehydrogenase (KDH), cytochrome b_2 (cyt. b_2) and cytochrome c_1 (cyt. c_1). (**B**) Parallel import reactions were carried out with the full-length (wild type) cytochrome c_1 precursor and the truncated version pc_1-Δ71C. Import was for 20 min at 12°C as described (Glick *et al.*, 1992), with ATP on both sides of the inner membrane, but no added NADH. After import the mitochondria were treated with trypsin (0.25 mg/ml for 15 min on ice) followed by soybean trypsin inhibitor (0.5 mg/ml). One aliquot of the mixture was removed (M). A second aliquot was converted to mitoplasts in the presence of proteinase K (MP). A third aliquot was treated with Na_2CO_3 and separated into supernatant (S) and membrane pellet (P) fractions. A fourth aliquot was supplemented with 2 mM NADH and 10 μM FMN, and subjected to a chase incubation for 30 min at 25°C in the presence of reticulocyte lysate (Nicholson *et al.*, 1989). One portion of each sample was then processed for SDS–PAGE and fluorography; a second portion was used for immunoblotting with antibodies against α-ketoglutarate dehydrogenase (KDH), cytochrome b_2 (cyt. b_2) and outer membrane porin. The 100% level for each marker protein was defined as the average of the values determined for the M and chase samples. STD, 20% precursor standards. p′ and i′, precursor and intermediate forms of pc_1-Δ71C; this protein was not processed to the mature form. (**C**) After synthesis of pre-cytochrome c_1, the reticulocyte lysate was spin-desalted through a 1 ml Sephadex G-25 column equilibrated in 0.1 M KCl, 20 mM potassium–HEPES (pH 7.4), 5 mM $MgCl_2$, 0.5 mM ATP and 20 mM DTT to remove precursors of heme. Import (15 min at 15°C), trypsin treatment, mitoplast generation and Na_2CO_3 extraction were as in panel B. Two aliquots of the mixture were subjected to a chase incubation for 20 min at 30°C, one of them (+ heme) in the presence of 2 mM NADH, 10 μM FMN and 2.5 μM hemin. A portion of each sample was subjected to SDS–PAGE and fluorography. A second portion was used for immunoblotting; the results were comparable to those given in B (not shown). STD, M, MP, S and P, as in B.

methods) in the presence of ATP, only ~10% of the molecules were imported into the matrix (Figure 5B, PK + Pel); the remainder were converted to the mature form, which was released into the incubation medium (Sup). It seems that the presequence of pc_1(1–64,R44)–DHFR had inserted into the mitoplast inner membrane, bringing the second cleavage site into contact with the processing protease and allowing cleavage to occur before the protein had a chance to be fully imported. Under these conditions the maturation of pc_1(1–64,R44)–DHFR was as efficient as that of the wild type fusion protein (Figure 5B). Thus in the ATP-depleted mitochondria, pc_1(1–64,R44)–DHFR accumulated as the intermediate-sized form because it was not positioned correctly for the second cleavage reaction. These results support the stop–transfer model.

Discussion

Since protein import across the inner membrane requires matrix ATP, a precursor that follows its normal import pathway in ATP-depleted mitochondria does not pass through the matrix. We found that depleting intramitochondrial ATP has no observable effect on the import and assembly of the adenine nucleotide translocator, suggesting that this protein inserts into the inner membrane from the external side. Import of the translocator does require ATP outside the mitochondria, indicating that the precursor interacts with a cytosolic chaperone(s). These results are consistent with reports that ATP is needed for insertion of the translocator into the outer membrane, but not for its subsequent integration into the inner membrane (Pfanner *et al.*, 1987), and that assembly of the translocator does not require hsp60 (Mahlke *et al.*, 1990).

The import pathway of cytochrome c_1 is more controversial. Based on results presented here and elsewhere (van Loon and Schatz, 1987; Glick *et al.*, 1992), we propose the scheme shown in Figure 6. ATP outside the mitochondria is required, probably for release of the precursor from an hsp70-type chaperone (step 1). During import, the intermembrane space-targeting domain in the presequence is recognized as a stop–transfer signal for the inner membrane. The mature domain of the protein then crosses the outer membrane and folds in the intermembrane space (step 2), allowing the hydrophobic sequence near the C-terminus to insert into the inner membrane (step 3). After addition of heme, the intermediate form is processed to the mature protein (step 4), which remains membrane-bound.

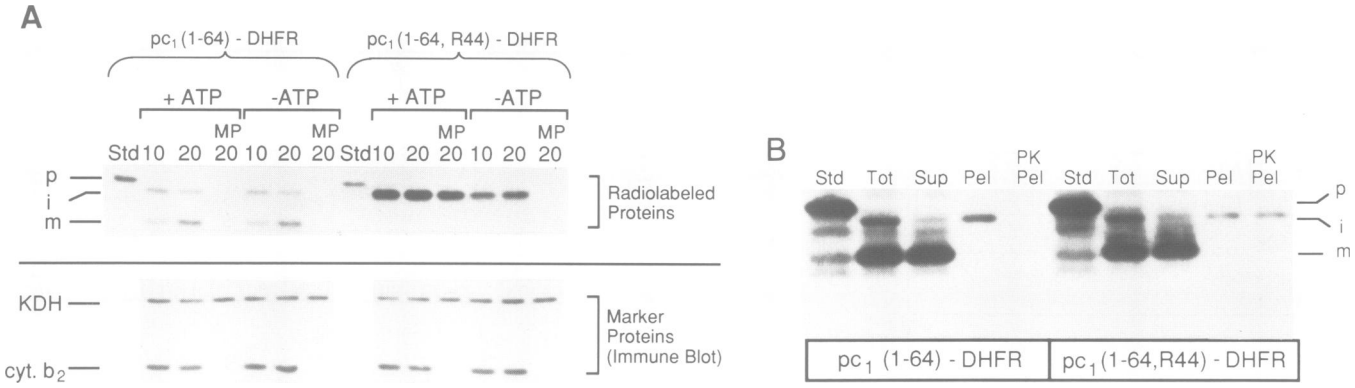

Fig. 5. $pc_1(1-64,R44)-DHFR$ is not processed to the mature form in ATP-depleted mitochondria even though it contains a functional second cleavage site. p, i and m, precursor, intermediate and mature forms of the fusion proteins. **(A)** $pc_1(1-64)-DHFR$ and $pc_1(1-64,R44)-DHFR$ were incubated with energized (+ATP) or ATP-depleted (−ATP) mitochondria. Import conditions and subsequent manipulations were as described (Glick *et al.*, 1992), except that the reactions contained 2 μg/ml efrapeptin, but no oligomycin. Import was for 10 or 20 min at 30°C. Aliquots were then treated with proteinase K, or converted to mitoplasts (MP) in the presence of proteinase K. A portion of each aliquot was taken for SDS−PAGE and fluorography (Std: 5% precursor standards). A second portion was used for immunoblotting with antibodies against α-ketoglutarate dehydrogenase (KDH) and cytochrome b_2 (cyt. b_2). **(B)** $pc_1(1-64)-DHFR$ and $pc_1(1-64,R44)-DHFR$ were incubated with mitoplasts in import buffer containing ATP (Glick *et al.*, 1992) for 10 min at 30°C. For one aliquot (Tot), the entire reaction mixture was precipitated with trichloroacetic acid and subjected to SDS−PAGE and fluorography. A second aliquot was centrifuged and separated into supernatant (Sup) and membrane pellet (Pel) fractions. A third aliquot was treated with proteinase K (PK) before centrifugation. Std: 100% precursor standards. The mature form of $pc_1(1-64)-DHFR$ generated by mitoplasts had the same electrophoretic mobility as the mature form in whole mitochondria, indicating that the same protease was involved (not shown).

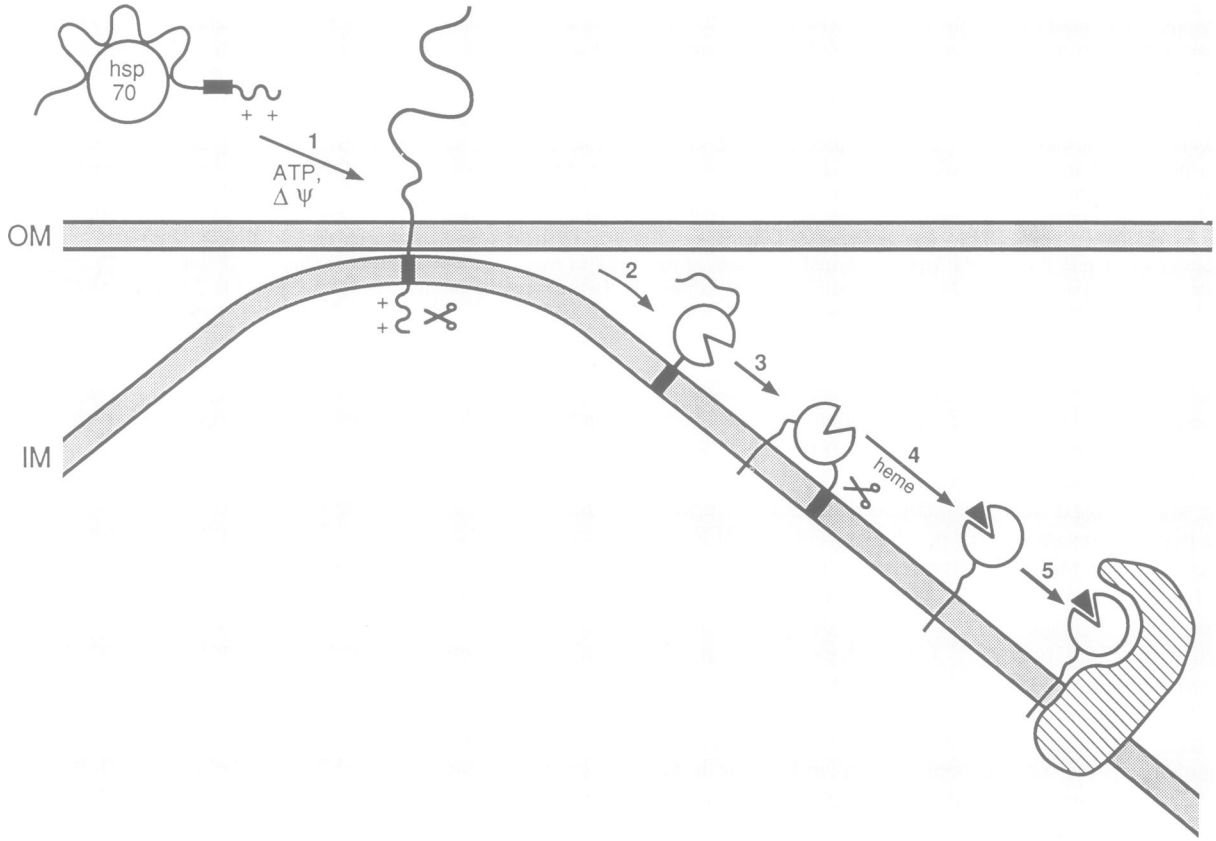

Fig. 6. Suggested import pathway of cytochrome c_1. The long wavy line represents the mature portion of the precursor; the presequence contains a domain that resembles a matrix-targeting signal (wavy line with + signs) followed by a stop−transfer sequence (black rectangle). (1) ATP-dependent release from cytosolic hsp70 followed by membrane insertion of the presequence. ΔΨ: electrochemical potential across the inner membrane. (2) Cleavage to the intermediate form, import to the intermembrane space, and folding of the apocytochrome domain. (3) Insertion of the C-terminal hydrophobic sequence into the inner membrane. (4) Attachment of heme (black triangle) followed by cleavage to the mature form. (5) Assembly into the cytochrome bc_1 complex.

Finally, cytochrome c_1 assembles with its partner subunits into the cytochrome bc_1 complex (step 5).

Except for the final assembly step, all of these reactions of the cytochrome c_1 import pathway are observed in our *in vitro* system and they occur with the same efficiency in ATP-depleted mitochondria as in fully energized

mitochondria. We interpret these data as evidence that cytochrome c_1 does not pass through the matrix on its way to the intermembrane space. The conservative sorting model could explain these results by postulating that ATP depletion traps cytochrome c_1 on its way into the matrix and that this non-physiological translocation intermediate then undergoes the reactions that would ordinarily take place only after re-export. Although this explanation cannot be ruled out, we feel it is unlikely for three reasons. (i) Cytochrome c_1 and the fusion protein $pc_1(1-64)-$DHFR are imported to the intermembrane space at similar rates in fully energized and in ATP-depleted mitochondria. It would be unusual for a non-physiological pathway to be as efficient as the normal pathway. For example, matrix-targeted proteins can also be imported across the outer membrane after ATP depletion, but the import rates are typically 3- to 10-fold lower than in the presence of ATP (Figures 2A and B, 3A and 5A; Hwang *et al.*, 1991; Glick *et al.*, 1992; C.Wachter and B.S.Glick, in preparation). (ii) Cytochrome c_1 and $pc_1(1-64)-$DHFR are cleaved to the mature form in ATP-depleted mitochondria. The same phenomenon is observed with a fusion protein that contains the cytochrome b_2 presequence (Glick *et al.*, 1992). Such data are readily explained if these proteins follow a stop–transfer pathway that is unaffected by depleting matrix ATP. According to the conservative sorting model, it is only by coincidence that ATP depletion arrests translocation of all of these proteins at a stage where processing can occur. This explanation seems improbable, as the results shown in Figure 5 suggest that an intermediate arrested by ATP depletion would not be correctly positioned for the second cleavage reaction. (iii) If cytochrome c_1 followed an import–re-export pathway, its hydrophobic C-terminal anchor sequence would presumably become integrated into the inner membrane during re-export from the matrix. However, this anchor sequence apparently still inserts into the inner membrane in ATP-depleted mitochondria, indicating that insertion can occur from the intermembrane space. The conservative sorting model thus has to postulate a second coincidence, that cytochrome c_1 uses a non-physiological insertion mechanism when import is arrested by ATP depletion.

The stop–transfer model does not specify how this C-terminal anchor sequence inserts into the inner membrane. This reaction may be catalysed by a protein or it may occur spontaneously. The peptide downstream of the anchor sequence contains a large number of positive charges (Sadler *et al.*, 1984) and resembles a mitochondrial matrix-targeting signal (Schatz, 1987), suggesting that it might cross the inner membrane by an electrophoretic mechanism (Pfanner and Neupert, 1985; Roise *et al.*, 1986).

The import pathway of cytochrome b_2 is probably similar to that of cytochrome c_1 (Hartl *et al.*, 1987; Glick *et al.*, 1992). However, we find that import of cytochrome b_2 requires ATP in the matrix as well as outside the mitochondria (Hwang *et al.*, 1989; C.Wachter and B.S.Glick, in preparation). While these results are consistent with the conservative sorting model, they can also be explained by the stop–transfer model, since matrix ATP might be needed to 'pull' the cytochrome b_2 precursor far enough into the mitochondria for the stop–transfer signal to be recognized (C.Wachter and B.S.Glick, in preparation).

If mhsp70 is indeed the ATP-dependent 'import motor' in the matrix, then our depletion experiments would suggest that mhsp70 function is not required for import of either the translocator or cytochrome c_1. It is therefore surprising that the import of these two precursors was blocked by inactivation of a temperature-sensitive mhsp70 protein (Ostermann *et al.*, 1990). The resolution of this interesting discrepancy should further our understanding of the mechanism of protein translocation.

Materials and methods

General methods

Most procedures have been described elsewhere (Glick, 1991; Glick *et al.*, 1992), including preparation of yeast mitochondria and measurement of mitochondrial protein concentration, import reaction conditions, *in vitro* synthesis of ^{35}S-labelled precursor proteins, osmotic shock to generate 'mitoplasts' (mitochondria whose outer membrane has been ruptured while the inner membrane remains intact), proteinase K treatment (50–100 μg/ml protease, 20–30 min on ice), use of protease-sensitive markers for the matrix (α-ketoglutarate dehydrogenase) and intermembrane space (cytochrome b_2), Na_2CO_3 extraction, precipitation with trichloroacetic acid, SDS–PAGE, fluorography, laser densitometry and immunoblotting with rabbit antisera and [^{125}I]protein A. To eliminate signals from ^{35}S-labelled proteins during immunoblotting, an additional sheet of X-ray film was included between the nitrocellulose filter and the film that was to be developed. Unless otherwise stated, mitochondria were re-isolated after the import reactions by centrifuging 5 min at 12 000 *g* and were resuspended in import buffer containing 5–10 μg/ml oligomycin, 1 μg/ml efrapeptin and 25 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP). Where indicated, the mitochondria were then treated with protease and/or converted to mitoplasts. Finally, the organelles were reisolated once again and precipitated with trichloroacetic acid. If detergent was present during protease treatment, trichloroacetic acid was added to the mixture directly.

ATP depletion procedures

The theoretical basis for these manipulations has been described (Glick, 1991). Apyrase (Sigma, Grade VIII) hydrolyses ATP and ADP to AMP. Oligomycin and efrapeptin are inhibitors of the mitochondrial ATP synthetase. Carboxyatractyloside (CAT) binds to the adenine nucleotide translocator and prevents ATP and ADP from crossing the inner membrane. For each procedure described below, the control conditions with fully energized mitochondria ('ATP in + out') were chosen to be as similar as possible to the ATP depletion conditions. These methods gave very reproducible results: complete translocation of matrix-targeted precursors across the inner membrane was typically inhibited >90% by depleting matrix ATP; and import of cytochrome c_1 and the adenine nucleotide translocator was consistently reduced >90% by depleting external ATP.

ATP out. Matrix ATP was selectively depleted. Mitochondria (1 mg/ml protein, in import buffer) were incubated for 5 min at 30°C with 10 U/ml apyrase, 12.5 μg/ml oligomycin and 1 μg/ml efrapeptin. CAT was then added to 50 μg/ml. After 5 min on ice, apyrase was removed by centrifuging the mitochondria through a cushion of 1.2 M sorbitol, 50 mM potassium HEPES (pH 7.4), 20 mM KCl, 10 mM potassium phosphate (pH 7.4) and 10 mM $MgCl_2$. The mitochondria were resuspended to 0.25 mg/ml in import buffer containing 12.5 μg/ml oligomycin, 1 μg/ml efrapeptin, 20 μg/ml CAT and 2 mM NADH. After a 3 min preincubation at the import temperature, 0.5 mM ATP and an ATP regenerating system (9 mM creatine phosphate and 0.1 mg/ml creatine kinase) were added, followed by the radiolabelled precursor. For the control with fully energized mitochondria, CAT was added before the 30°C preincubation and oligomycin and efrapeptin were omitted throughout the procedure.

ATP in. External ATP was selectively depleted. Precursors in reticulocyte lysate were depleted of ATP with 50 U/ml glycerokinase (from *Candida*) plus 100 mM glycerol for 5 min at 30°C; this treatment did not cause degradation of any of the precursors used (not shown). Mitochondria (0.25 mg/ml in import buffer) were incubated with 50 μg/ml CAT for 5 min on ice; NADH (2 mM) and apyrase (10 U/ml) were then added and the mixture was incubated for 5 min at 30°C and 3 min at the import temperature before addition of the glycerokinase-treated precursor. For the control with fully energized mitochondria, glycerokinase was omitted and 1 mM ATP plus an ATP-regenerating system was substituted for apyrase. As previously reported (Hwang and Schatz, 1989), depletion of external ATP did not affect import of the matrix-targeted precursor pCOXIV–DHFR (not shown).

No ATP or –ATP. ATP was depleted on both sides of the inner membrane. Precursors were treated with glycerokinase as above. Mitochondria (0.25 mg/ml in import buffer) were incubated for 5 min at 30°C with 10 U/ml apyrase, 12.5 μg/ml oligomycin and 1 μg/ml efrapeptin. NADH (2 mM) was then added and the mixture was incubated 3 min at the import

temperature before addition of the glycerokinase-treated precursor. For the control with fully energized mitochondria, glycerokinase was omitted and 1 mM ATP plus an ATP-regenerating system was substituted for apyrase.

Incubation of fusion proteins with mitoplasts

Mitoplasts were prepared as previously described by Ohba and Schatz (1987) and Hwang *et al.* (1989), except that mitochondria were not treated with protease before the osmotic shock. Protease pretreatment was unnecessary because with $pc_1(1-64)$−DHFR and $pc_1(1-64,R44)$−DHFR, direct insertion into the mitoplast inner membrane is much faster than import into whole mitochondria (B.S.Glick and K.Cunnungham, in preparation). When the wild type fusion protein $pc_1(1-64)$−DHFR is incubated with energized mitoplasts, the intermediate form becomes bound to the outer surface of the inner membrane, as in intact mitochondria; the mature form is released into the incubation medium, which is equivalent to the intermembrane space.

Terminology

(See Hwang and Schatz, 1989.) Although we refer only to the depletion of ATP, our procedures should also cause hydrolysis of GTP and other nucleoside triphosphates. For convenience, we use the term 'ATP-depleted mitochondria' to mean that the matrix was depleted of ATP, regardless of whether ATP was present outside the inner membrane. 'Intramitochondrial ATP' means ATP in the matrix; 'external ATP' or 'ATP outside the mitochondria' refers to ATP outside the inner membrane, including ATP that may be present in the intermembrane space.

Miscellaneous

Carboxyatractyloside was purchased from Fluka and efrapeptin was a gift of the Eli Lilly Corporation; other reagents were from Sigma. The plasmid pc_1-Δ71C, which encodes a cytochrome c_1 precursor lacking the C-terminal 71 amino acids (Hase *et al.*, 1987), was constructed by linearizing plasmid 1 (van Loon *et al.*, 1986) with *Kpn*I, generating blunt ends with T4 polymerase and religating. Plasmid 9/2 (van Loon *et al.*, 1986), which encodes $pc_1(1-64)$−DHFR, was modified by PCR mutagenesis (Ho *et al.*, 1989): Ala44 (codon GCC) of the presequence was changed to arginine (CGC) to give the mutant precursor $pc_1(1-64,R44)$−DHFR. The plasmid encoding pSu9(1−69)−DHFR was kindly provided by Dr Walter Neupert.

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