Targeting and translocation of proteins into the hydrogenosome of the protist *Trichomonas*: similarities with mitochondrial protein import

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Trichomonads are early-diverging eukaryotes that lack both mitochondria and peroxisomes. They do contain a double membrane-bound organelle, called the hydrogenosome, that metabolizes pyruvate and produces ATP. To address the origin and biological nature of hydrogenosomes, we have established an *in vitro* protein import assay. Using purified hydrogenosomes and radiolabeled hydrogenosomal precursor ferredoxin (pFd), we demonstrate that protein import requires intact organelles, ATP and *N*-ethylmaleimide-sensitive cytosolic factors. Protein import is also affected by high concentrations of the protonophore, *m*-chlorophenylhydrazine (CCCP). Binding and translocation of pFd into hydrogenosomes requires the presence of an eight amino acid N-terminal presequence that is similar to presequences found on all examined hydrogenosomal proteins. Upon import, pFd is processed to a size consistent with cleavage of the presequence. Mutation of a conserved leucine at position 2 in the presequence to a glycine disrupts import of pFd into the organelle. Interestingly, a comparison of hydrogenosomal and mitochondrial protein presequences reveals striking similarities. These data indicate that mechanisms underlying protein targeting and biogenesis of hydrogenosomes and mitochondria are similar, consistent with the notion that these two organelles arose from a common endosymbiont.

Keywords: hydrogenosome/organelle evolution/protein translocation/*Trichomonas*

Introduction

Compartmentalization of specialized cellular functions into organelles is a characteristic of all eukaryotic cells. Some organelles, such as mitochondria and peroxisomes, are common to most eukaryotes. There are also a number of unusual organelles, such as the hydrogenosome of trichomonads and the glycosome of trypanosomatids, that exist primarily in very early-evolving eukaryotes. Interestingly, eukaryotes that possess these organelles typically lack either mitochondria or peroxisomes or both, raising questions regarding the origin of these unusual organelles.

The hydrogenosome is found exclusively in organisms that lack mitochondria. These organisms comprise a broad

phylogenetic range and include anaerobic, rumen-dwelling ciliates and fungi, as well as free-living ciliates (Yarlett *et al.*, 1981, 1986; van Bruggen *et al.*, 1984; Lloyd *et al.*, 1989; Paul *et al.*, 1990; Fenchel and Finlay, 1991; O'Fallon *et al.*, 1991; Marvin-Sikkema *et al.*, 1994). The hydrogenosome has been best studied in the flagellated parasitic protist, *Trichomonas vaginalis* (Johnson *et al.*, 1995).

As the site of pyruvate fermentation in trichomonads, hydrogenosomes play a central role in carbohydrate metabolism of these amitochondriate protists. Within the organelle, pyruvate is metabolized to the end-products of acetate, CO₂ and molecular hydrogen. This process is coupled to ATP formation via substrate-level phosphorylation. In this regard, hydrogenosomes can be considered the anaerobic equivalent of mitochondria. Although biochemical analyses of hydrogenosomes reveal properties similar to those of mitochondria, there are also significant differences. For example, the enzyme that mediates decarboxylation of pyruvate in hydrogenosomes, pyruvate:ferredoxin oxidoreductase, is completely different from its counterpart in mitochondria, the pyruvate dehydrogenase complex. Unlike mitochondria, hydrogenosomes contain hydrogenases and produce molecular hydrogen and do not contain cytochromes, DNA or ribosomes (Lloyd *et al.*, 1979b; Müller, 1993; Johnson *et al.*, 1995). Hydrogenosomes are similar to mitochondria in their abilities to metabolize pyruvate and produce ATP. The enzyme that catalyzes substrate-level production of ATP in hydrogenosomes, succinyl CoA synthetase, is in fact located exclusively in mitochondria in higher eukaryotic cells. Hydrogenosomes have also been demonstrated to contain a [2Fe-2S]-ferredoxin that is structurally similar to mitochondrial ferredoxins (Gorrell *et al.*, 1984; Johnson *et al.*, 1990). Although bounded by a double membrane, the inner membrane of the hydrogenosome does not form cristae as observed in mitochondria (Honigberg *et al.*, 1984).

It was hypothesized originally that hydrogenosomes arose from an endosymbiotic anaerobic bacterium (Whatley *et al.*, 1979; Müller, 1980), in view of their anaerobic metabolism and the presence of pyruvate:ferredoxin oxidoreductase and hydrogenase, two enzymes that are commonly found in methanogens and fermentative anaerobic bacteria. An opposing hypothesis, which took into account the structural and biochemical similarities between hydrogenosomes and mitochondria, proposed that hydrogenosomes are converted mitochondria (Cavalier-Smith, 1987). Recent evolutionary analyses of rRNA sequences demonstrate that trichomonads diverged from the main line of eukaryotic evolution prior to the advent of authentic mitochondria (Viscogliosi *et al.*, 1993; Gunderson *et al.*, 1995). This brings forth the intriguing possibility that mitochondria and hydrogenosomes evolved from a common endosymbiont or progenitor organelle

Table I. N-terminal presequences of hydrogenosomal proteins

Hydrogenosomal protein	Presequence	▼	Reference
Ferredoxin	MLSQVCRF	GTIT	Johnson <i>et al.</i> (1990)
β -Succinyl-CoA synthetase 1	MLSASSNFARN	FNIL	Lahti <i>et al.</i> (1992)
β -Succinyl-CoA synthetase 53	MLSSSFARN	FNIL	Johnson <i>et al.</i> (1993)
α -Succinyl-CoA synthetase 1	MLAGDFSRN	LKOP	Lahti <i>et al.</i> (1994)
α -Succinyl-CoA synthetase 2	MLSSSFERN	LHQP	Lahti <i>et al.</i> (1994)
α -Succinyl-CoA synthetase 3	MLSSSFERN	LHQP	Lahti <i>et al.</i> (1994)
Adenylate kinase	MLSGVSRN	AART	Lange <i>et al.</i> (1994)
Pyruvate:ferredoxin oxidoreductase 1	MLRNF	SKRV	Hrdy and Müller (1995a)
Pyruvate:ferredoxin oxidoreductase 2	MLRSF	GKRI	Hrdy and Müller (1995a)
Malic enzyme 1	MLTSSVSVPRN	ICRA	Hrdy and Müller (1995b)
Malic enzyme 2	MLTSVSYPRN	ICRS	Hrdy and Müller (1995b)
Hsp60	MSLIEAAKHFTRAF	AKAR	Bui <i>et al.</i> (1996)

Presequences are defined as sequences encoded by the gene, but absent from the N-terminus upon N-terminal sequencing of the protein isolated from the organelle. The presequence and first four amino acids of the mature protein are shown, separated by a triangle marking the cleavage site.

(Johnson *et al.*, 1995), a refinement of the earlier hypothesis that hydrogenosomes were derived by conversion of authentic mitochondria.

To address the origin of hydrogenosomes and determine their relationship to mitochondria, the biogenesis of hydrogenosomes has been examined. We have demonstrated that *T.vaginalis* hydrogenosomal proteins are synthesized on free polyribosomes and thus appear to contain topogenic signals for post-translational targeting and translocation into the organelle (Lahti and Johnson, 1991). Here we describe the development of an *in vitro* hydrogenosomal import assay. Using this assay, we have determined biochemical properties that are essential for the targeting and translocation of proteins into the hydrogenosome and have established that a conserved N-terminal presequence found on all examined hydrogenosomal proteins is a targeting signal that is necessary for protein import into the organelle.

Results

Hydrogenosomal presequences

Characterization of 12 *T.vaginalis* genes that encode hydrogenosomal proteins and N-terminal sequence analysis of these proteins (Johnson *et al.*, 1990, 1993; Lahti *et al.*, 1992, 1994; Lange *et al.*, 1994; Hrdy and Müller, 1995a,b; Bui *et al.*, 1996) has invariably revealed the presence of short, N-terminal presequences that are encoded in the genes but are absent from the mature proteins. These presequences are strikingly similar to each other (Table I). Eleven out of the 12 presequences have leucine at position 2. Arginine is present at either -2 or -3 from the cleavage site, and asparagine or phenylalanine is present at -1 from the cleavage site. These sequences are also rich in hydrophobic and hydroxylated amino acids. The fact that these hydrogenosomal proteins are synthesized on free polysomes in the cytosol (Lahti and Johnson, 1991), with similar presequences which are then removed from the mature proteins, indicates that the presequences may function in targeting. To test this hypothesis, we have developed an *in vitro* import assay using radiolabeled precursor proteins and purified hydrogenosomes.

Isolated hydrogenosomes are highly purified and intact

In order to examine *in vitro* import into hydrogenosomes, we have isolated a *T.vaginalis* subcellular fraction that contains hydrogenosomes with minimal contamination of other membrane-bound organelles. To separate hydrogenosomes from lysosomes and other organelles, a post-nuclear supernatant (PNS) was subjected to centrifugation in 45% Percoll, 0.25 M sucrose. This procedure, a modification of that described by Opperdoes and colleagues for purification of glycosomes (Opperdoes *et al.*, 1984), clearly separates hydrogenosomes from lysosomes. Hydrogenosomes band in the bottom third of the gradient (Figure 1A, fraction 4), whereas lysosomes, which are less dense than hydrogenosomes (Lockwood *et al.*, 1988), band in the top third of the gradient (fraction 2). The five fractions illustrated in Figure 1A were collected from the gradient and washed to remove soluble contaminants. The fractions were then assayed for the presence of the hydrogenosomal marker enzyme, malate dehydrogenase (decarboxylating) (MDH) (Steinbüchel and Müller, 1986) (Figure 1B) and the lysosomal marker enzyme, acid phosphatase (Müller, 1973) (Figure 1C). The majority of acid phosphatase activity (5.3%) appears in fraction 2, with very little contamination from MDH activity. The poor overall recovery of this lysosomal marker is expected as lysosomes do not pellet efficiently at 7500 g and are lost in the wash steps. The majority of MDH activity (38.6%) appears in fraction 4, which contains the hydrogenosomes. These hydrogenosomes are intact as assessed by latency of MDH in this fraction, which is 88% (data not shown). The amount of recovered acid phosphatase activity in fraction 4 is 0.7%. Thus, our purified hydrogenosome preparation is intact and contains negligible lysosomal contaminants.

The homogeneity of our hydrogenosomal preparation is also demonstrated by examination by electron microscopy. Figure 2A shows a transmission electron micrograph (TEM) of a thin section through the hydrogenosome fraction used in import reactions. This preparation contains a homogenous population of organelles of medium electron density, ~0.8 μ m in diameter. Many of these organelles possess an electron-dense inclusion located at one end. Although the function of these inclusions has not been determined, analysis of hydrogenosome preparations by

Fig. 1. Percoll gradient purification of hydrogenosomes. (A) Photograph of separation of hydrogenosomes and lysosomes on a 45% Percoll gradient. The numbers indicate fractions taken for marker enzyme assays. (B and C) Distribution of marker enzymes recovered in washed fractions after equilibrium density gradient centrifugation in 45% Percoll in SMD. The area under each histogram is proportional to the percentage of total enzyme activity present in the starting material. (B) Malate dehydrogenase (decarboxylating) (MDH), a marker enzyme for hydrogenosomes (Steinbüchel and Müller, 1986); (C) acid phosphatase (AP), a marker enzyme for lysosomes (Müller, 1973).

energy-dispersive X-ray microanalysis indicates an accumulation of Mg^{2+} , PO_4^- and Ca^{2+} (Chapman *et al.*, 1985). Higher magnification of this preparation reveals the double membrane which surrounds the hydrogenosome (Figure 2B).

pFd is imported into intact hydrogenosomes and processed to the mature size

We have established an *in vitro* hydrogenosomal import assay consisting of purified organelles, *T.vaginalis* cytosol, and radiolabeled precursor proteins. These components are incubated in buffer that stabilizes hydrogenosomes and provides an ATP-regenerating environment. This assay system is similar to those established for *in vitro* import into isolated mitochondria (Gasser *et al.*, 1982). To monitor import activity, we have used the iron-sulfur protein, ferredoxin (Johnson *et al.*, 1990), as a precursor. The small size of this protein readily allows discrimination of the precursor (103 amino acids) and the mature protein (95 amino acids) by 17.5% SDS-PAGE. Following incubation of the radiolabeled ferredoxin precursor (pFd) with purified hydrogenosomes and *T.vaginalis* cytosol at 37°C for 30 min, the organelles were washed by centrifugation

Fig. 2. Electron micrographs of thin sections of Percoll gradient-purified hydrogenosomes (see Materials and methods). (A) Field showing a homogeneous preparation of hydrogenosomes, magnified 7500 times. (B) Sectioned hydrogenosomes showing double membrane, magnified 75 000 times.

and re-isolated to remove unbound radiolabeled pFd. To assess the location of radiolabeled protein in washed hydrogenosomes, we examined the accessibility of the protein to added protease in the absence or presence of detergent lysis. In samples where proteinase K was not added, pFd associates with the organelles (Figure 3, lane 2), and is mostly processed to a smaller size (Fd). The size of Fd is the same as Δ Fd, a protein from which the presequence has been deleted (Figure 3, lane 9). When proteinase K is added, the faint upper band corresponding to pFd is digested, whereas Fd is protease protected (lane 3). Digestion of only pFd in proteinase K-treated samples indicates that the precursor is on the outside of the organelle while Fd is inside. The protease protection of Fd is membrane-conferred, as disruption of the membrane by the addition of the non-ionic detergent Triton X-100 results in digestion by proteinase K (lane 4).

To determine if intact organelles are required for protease protection and cleavage of pFd, we have tested organelles disrupted by sonication. A negligible amount of pFd associates with sonicated hydrogenosomes (Figure 3, lane 6), and no protease-protected form is detected (lane 7), showing that import requires intact organelles. In mock import reactions carried out in the absence of hydrogenosomes, the precursor remains full length and soluble (data not shown).

These results, taken together, indicate that pFd associates with hydrogenosomes *in vitro* and is translocated into a

Fig. 3. Import of radiolabeled pFd into purified hydrogenosomes and requirement for intact organelles. Purified hydrogenosomes were incubated with radiolabeled pFd. Unbound precursor was removed by re-isolating and washing the organelles. The uptake of the pFd was assessed by proteinase K treatment in the absence or presence of detergent lysis (see Materials and methods). Samples were analyzed by 17.5% SDS-PAGE and fluorography. Lane 1, precursor Fd (pFd, 10% of assay input); 2, untreated sample; 3, proteinase K-treated sample; 4, proteinase K- and Triton X-100-treated sample; 5, same as lane 1; 6–8 are the same as 2–4 except using sonicated hydrogenosomes in the import reaction; 9, radiolabeled Δ Fd loaded as a size marker for the migration of ferredoxin lacking a presequence.

Fig. 4. Import of pFd into purified hydrogenosomes is dependent on ATP and temperature. Lanes 1, 5 and 9, pFd (10% of assay input); 2–4, pFd import reaction at 37°C and protease protection assay; 6–8, pFd import reaction at 0°C; 10–12, pFd import reaction in the absence of ATP. ATP was removed from the assay by addition of apyrase to 200 U/ml and exclusion of ATP, ADP and an ATP-regenerating system.

protease-protected compartment within the organelle where it is proteolytically processed to a size consistent with the removal of the presequence.

Import is temperature and ATP dependent

To examine the energy requirements for pFd import, we tested whether *in vitro* import was dependent on temperature and ATP (Figure 4). Binding of pFd to hydrogenosomes is reduced at 0°C and no processed form of the protein is detected. Bound pFd is not protected from externally added protease, indicating that it resides outside the organelle and is not translocated (Figure 4, lanes 6–8). Temperature-dependent translocation of proteins indicates an active process, and is similar to that observed for *in vitro* translocation into other organelles (Daum *et al.*, 1982; Hurt *et al.*, 1984; Imanaka *et al.*, 1987).

We tested the requirement for ATP in *in vitro* import into hydrogenosomes by depleting the reaction of ATP by using apyrase and excluding the ATP-regenerating system from the assay (Figure 4). Under these conditions, pFd binds to isolated hydrogenosomes but only a small portion is cleaved and protease protected (Figure 4, lanes 10–12). The small amount that is translocated could be due to residual ATP. These data show that pFd binds to isolated hydrogenosomes under ATP-deficient conditions but is not translocated, consistent with a requirement for ATP hydrolysis for protein translocation across membranes (Goldfarb, 1992).

Fig. 5. Import is sensitive to the protonophore CCCP. Lanes 1–6, import in the presence of 0–1000 μM CCCP, untreated samples; 7, import in the presence of 1000 μM CCCP, proteinase K-treated sample; 8, import in the absence of ATP, untreated sample; 9, import in the absence of ATP, proteinase K-treated sample, demonstrating the different proteinase K sensitivity of pFd in CCCP-treated (lane 7) or ATP-depleted (lane 9) import reactions.

Import is sensitive to the protonophore CCCP

Hydrogenosomes have been reported to generate a transmembrane electrochemical potential (Humphreys *et al.*, 1994). As the existence of an electrochemical potential tends to correlate with a role in protein translocation across membranes (Schleyer *et al.*, 1982; Bakker and Randall, 1984; Robinson *et al.*, 1993), we investigated whether the presence of the protonophore *m*-chlorophenylhydrazine (CCCP) in the *in vitro* import assay affects import. Figure 5 shows that import, as judged by conversion of pFd to Fd, decreases at 200 μM CCCP and is completely abolished at 600 μM CCCP. However, unlike import, binding of pFd to isolated hydrogenosomes increases with increasing levels of CCCP. Proteinase K treatment of import reactions conducted in the presence of CCCP shows that ~40% of pFd is resistant to 100 μg/ml proteinase K (lane 7). This partial resistance of bound pFd to proteinase K is only observed at CCCP concentrations that block import, while pFd bound to the organelle in the absence of ATP is completely digested by proteinase K (lane 9). We therefore assume that the proteinase K-resistant precursor observed in the presence of CCCP represents an import intermediate which, in contrast to pFd bound to the organelle upon ATP depletion, resides in a protease-protected compartment. Although the effect of CCCP on hydrogenosomes is not known, these results suggest that a transmembrane electrochemical potential is necessary to complete translocation of proteins into hydrogenosomes.

Import requires NEM-sensitive cytosolic factors

Our standard *in vitro* import assays contain a *T.vaginalis* cytosolic fraction. Import assays carried out in the absence of cytosol show that translocation of pFd is dependent on the presence of the *T.vaginalis* cytosolic fraction (Figure 6A). In the absence of cytosol, the precursor binds to the organelle, but is not translocated as it is not protease protected or processed to the mature size (lanes 4–6). In contrast to the situation in other *in vitro* translocation systems (Murakami *et al.*, 1988), neither rabbit reticulocyte lysate nor wheat germ extract can substitute for *T.vaginalis* cytosol (data not shown).

To begin characterization of the cytosolic factors necessary for import, we treated the cytosol with the alkylating agent *N*-ethylmaleimide (NEM). NEM-sensitive factors have been shown to be involved in transport to various intracellular locations (Murakami *et al.*, 1988; Newmeyer

Fig. 6. Import is dependent on *T.vaginalis* cytosolic factors that are sensitive to NEM. (A) pFd import reactions carried out in the presence (lanes 1–3) or absence (lanes 4–6) of cytosol. (B) Lane 1, pFd (10% of assay input); 2–4, pFd control import reaction and protease protection assay; 5–7, pFd import with 10 mM NEM-treated cytosol; 6–8, pFd import with DTT-inactivated NEM demonstrating the necessity for the alkylation activity of NEM.

and Forbes, 1990; Wendland and Subramani, 1993a). Pre-incubation of the cytosol with 10 mM NEM abolishes translocation (Figure 6B, lanes 5–7), resulting in reactions similar to those lacking cytosol (Figure 6A). We tested the effect of inactivating NEM with 20 mM dithiothreitol (DTT) to verify that the loss of cytosolic factor activity was due to alkylation by NEM. DTT-inactivated NEM reactions (Figure 6B, lanes 8–10) were identical to minus-NEM import reactions (Figure 6B, lanes 2–4). In addition, heat treatment of the cytosol at 95°C for 10 min abolishes translocation (data not shown). These results suggest that the activity in the cytosol that is necessary for import into hydrogenosomes is due to protein component(s), at least one of which is sensitive to NEM.

Import is presequence dependent

To test whether import is dependent on the presence of the N-terminal presequence on pFd, we constructed ΔFd which lacks residues 2–8 of the precursor. In import reactions containing radiolabeled ΔFd, the protein lacking the presequence does not associate with the isolated hydrogenosomes and is not protease protected (Figure 7A, lanes 6–8), in contrast to that observed using precursor protein pFd (Figure 7A, lanes 2–4). Therefore, the binding and translocation of pFd depends on the presence of the presequence, since its removal abolishes association with the hydrogenosomes. These data show that information necessary for targeting the protein to the organelle resides within the presequence.

Fig. 7. (A) Import is dependent on the presence of the presequence. Lane 1, pFd (10% of assay input); 2–4, pFd control import reaction and protease protection assay; 5, ΔFd (10% of assay input); 6–8, ΔFd import reaction and protease protection assay. (B) Import is disrupted by a leucine to glycine substitution at position 2 of the ferredoxin presequence (pFdL2G). Lane 1, pFd size marker; 2, presequence-deleted ferredoxin (ΔFd) size marker; 3, pFdL2G (10% of assay input); 4–6, pFdL2G import reaction and protease protection assay.

A single amino acid change in the ferredoxin presequence can disrupt import

As shown in Table 1, 11 out of 12 hydrogenosomal presequences identified to date contain a leucine at the second position of the presequence. To test whether the high degree of conservation of this residue reflects a functional requirement for leucine at this position, we mutated this residue in the ferredoxin presequence to a glycine (pFdL2G). When pFdL2G is tested in the *in vitro* import assay, this single amino acid change is shown to nearly abolish import (Figure 7B). A small amount of the radiolabeled precursor does associate with the organelle and is protease protected, but is not cleaved to the mature size (lanes 4 and 5). This could be due to the mutant presequence not being properly recognized or cleaved by a protease responsible for cleaving imported precursor proteins. Alternatively, the small fraction that is transported could be inaccessible to the protease. These data demonstrate that glycine cannot substitute for leucine at position 2 of the ferredoxin presequence and that a single amino acid change in the presequence can inhibit binding and translocation.

Discussion

In vitro protein import

We have established an *in vitro* protein import assay to study the biogenesis and address the origin of the hydrogenosome, a specialized organelle found exclusively in early-evolving eukaryotes that lack mitochondria (Lindmark and Müller, 1973; Johnson *et al.*, 1993; Müller,

1993). Short N-terminal presequences found on all hydrogenosomal proteins examined to date have been proposed to serve as targeting signals (Johnson *et al.*, 1990, 1993; Lahti *et al.*, 1992, 1994; Lange *et al.*, 1994; Hrdy and Müller, 1995a); however, this hypothesis has not been tested functionally. Here, we demonstrate that the presequence of the hydrogenosomal matrix protein, ferredoxin (pFd), is necessary for import into intact hydrogenosomes isolated from *T.vaginalis*. Import is accompanied by processing to the size of the mature protein and requires intact organelles, ATP, a transmembrane electrochemical potential and cytosolic factors. Our studies also demonstrate the importance of a conserved leucine at position 2 in the presequence, as changing this residue to a glycine nearly abolishes binding and translocation of the protein into the organelle.

Many of the properties underlying protein import into the matrix of hydrogenosomes are similar to those previously observed for import of proteins into the mitochondrial matrix (Glick and Schatz, 1991; Cuezva *et al.*, 1993; Schwarz and Neupert, 1994). Transport into both organelles is dependent on an N-terminal, cleavable presequence, cytosolic factors, energy in the form of temperature and ATP, and is inhibited by the presence of the protonophore CCCP. In mitochondrial *in vitro* import assays, precursor proteins bind at low temperature, but translocation is blocked (Chien *et al.*, 1984). Translocation of pFd into hydrogenosomes is also completely inhibited at 0°C. Binding occurs at low temperatures, albeit at significantly reduced levels.

Import is ATP dependent

ATP is generally required for protein translocation across membranes (Goldfarb, 1992). Hydrogenosomal import reactions deficient in ATP show that pFd translocation is ATP dependent while binding of pFd to hydrogenosomes requires little or no ATP. These experiments demonstrate that hydrogenosomal import can be separated into discrete steps of ATP-deficient binding and ATP-dependent translocation. We cannot exclude the possibility that residual ATP levels may contribute to the observed binding. Proteins destined for internal chloroplast compartments require low ATP levels for initial binding, followed by high ATP levels for translocation (Olsen *et al.*, 1989). ATP levels that allow binding but are insufficient to support translocation have also been described for import of some mitochondrial precursors (Chien *et al.*, 1984). Although the role of ATP in protein translocation into hydrogenosomes is yet to be elucidated, analyses of protein import into other organelles (Wendland and Subramani, 1993b; Schwarz and Neupert, 1994; Schnell, 1995) suggest that ATP functions at several stages. ATP hydrolysis is required for maintaining precursors in an unfolded 'translocation-competent' state in the cytosol, the release of chaperones, translocation across membranes and the proper folding of newly translocated matrix proteins.

Effect of CCCP on import

The inhibitory effect of CCCP on the import of pFd is consistent with a requirement for a transmembrane electrochemical potential in addition to ATP for hydrogenosomal import. Nonetheless, definitive evidence that such a potential is necessary for translocation of proteins

into hydrogenosomes awaits a direct demonstration that dissipation of a gradient blocks translocation. Protein import into the mitochondrial matrix requires, in addition to ATP, a transmembrane electrochemical potential. Specifically, the membrane potential component $\Delta\Psi$ (Schleyer *et al.*, 1982; Pfanner and Neupert, 1985; Eilers *et al.*, 1987; Martin *et al.*, 1991; Ungermann *et al.*, 1996), which is generated in respiring mitochondria by proton translocation from the matrix across the inner membrane, is needed for import into mitochondria (Boyer *et al.*, 1977). Similarly, protein export across the bacterial plasma membrane also requires both ATP and a transmembrane electrochemical potential (Bakker and Randall, 1984; Schiebel *et al.*, 1991). In contrast, protein translocation across other cellular membranes requires either ATP (Waters and Blobel, 1986; Imanaka *et al.*, 1987; Newmeyer and Forbes, 1988; Brock *et al.*, 1995; Görlich and Mattaj, 1996) or the ΔpH component of the transmembrane electrochemical potential (Brock *et al.*, 1995) as an energy source. In the hydrogenosomal import reaction, it remains to be elucidated whether the total electrochemical potential Δp is utilized or whether either of its two components ΔpH or $\Delta\Psi$ alone is sufficient for hydrogenosomal import.

Hydrogenosomes lack cytochromes as well as an F_0F_1 -ATPase (Lloyd *et al.*, 1979a,b) and therefore seem to generate a membrane potential by a process other than respiration or ATP hydrolysis. This closely resembles the situation in ρ^- mutants in yeast. Despite a lack of respiration and a functional F_0F_1 -ATPase, these mutants are capable of processing mitochondrial proteins *in vivo* (Schatz and Mason, 1974). It is assumed that ρ^- mutants maintain a small membrane potential, possibly coupled to ATP-ADP exchange via the adenine nucleotide translocator (Klingenberg and Rottenberg, 1977). Generation of a comparably small membrane potential in hydrogenosomes would be sufficient for import of pFd, as the relatively high concentration of CCCP needed to inhibit import [$\sim 600 \mu M$ compared with $50\text{--}150 \mu M$ for respiring mitochondria (Martin *et al.*, 1991)] indicates the requirement for only a small potential (Martin *et al.*, 1991).

Interestingly, pFd bound to the hydrogenosome in the presence of CCCP and ATP is partially resistant to digestion with proteinase K. In contrast, when pFd is bound in the absence of ATP, a condition that restricts all translocation, the protein is completely digested. It is possible that the proteinase-protected precursor observed in the presence of CCCP is translocated into the organelle but is not cleaved due to an unknown effect of CCCP on the hydrogenosomal processing enzyme, although this seems unlikely. A more probable explanation for the protection of a precursor form in the presence of CCCP is that it represents a translocation intermediate which is buried in the membrane and therefore protected against digestion with proteinase K, similar to the stage 3 intermediate observed in mitochondrial protein import after the inner membrane is de-energized (Pfanner and Neupert, 1987).

Import is dependent on N-ethylmaleimide-sensitive cytosolic factors

Hydrogenosomal import requires the presence of NEM-sensitive *T.vaginalis* cytosolic factors. Transport of newly synthesized proteins to various subcellular locations has

been shown to require cytosolic proteins that are sensitive to NEM (Murakami *et al.*, 1988; Wendland and Subramani, 1993a). These factors are best characterized in mitochondrial import studies. Cytosolic Hsp70 prevents folding or aggregation of precursor proteins prior to transport (Komiya *et al.*, 1996). MSF (mitochondrial import-stimulating factor) binds mitochondrial presequences and directs precursors to mitochondrial surface receptors (Hachiya *et al.*, 1995). MSF also has an ATP-dependent unfoldase activity that dissociates aggregated precursor proteins (Hachiya *et al.*, 1993). Whether hydrogenosomal proteins utilize cytosolic factors that are similar to those used for mitochondrial import is as yet unknown; however, at least one of the factors required for import into both organelles is an NEM-sensitive protein.

Comparison of protein import into hydrogenosomes and mitochondria

A comparison of hydrogenosomal and mitochondrial protein presequences reveals many similarities, although there are also notable differences. Presequences of both hydrogenosomal and mitochondrial proteins are absolutely required for binding and translocation of proteins into the matrix of the organelle *in vitro*. Hydrogenosomal and mitochondrial targeting sequences are also similar as they both have an N-terminal location, are cleaved from the mature protein found in the organelle and have similar amino acid composition. For example, 92% of examined hydrogenosomal presequences begin with methionine-leucine and 50% begin with methionine-leucine-serine. By comparison, 40% of known mitochondrial presequences begin with methionine-leucine and 16% begin with methionine-leucine-serine (Hendrick *et al.*, 1989). In contrast, the N-terminal presequences that direct proteins into chloroplasts almost invariably begin with methionine-alanine (von Heijne *et al.*, 1989). Our data indicate a functional role for the conserved leucine residue at position 2 in hydrogenosomal presequences, as mutating this residue to a glycine abolishes nearly all detectable binding to the organelle. However, similarly to what is seen in mitochondrial presequences, there is not a strict requirement for leucine at this position, as the presequence of hydrogenosomal Hsp60 contains a serine in position 2 (Table I). Hydrogenosomal and mitochondrial presequences are similar in other aspects. Nine of the 12 characterized hydrogenosomal presequences contain arginine at -2 from the cleavage site and a majority of mitochondrial presequences contain arginine at either -2 or -10 from the cleavage site (Hendrick *et al.*, 1989; von Heijne *et al.*, 1989). Interestingly, the three hydrogenosomal presequences that do not have an arginine at -2 have this residue at -3 relative to the cleavage site. Arginine, leucine and serine typically are overrepresented in mitochondrial presequences and all three amino acids are present in 11 out of the 12 short hydrogenosomal presequences defined to date. Finally, both hydrogenosomal (Johnson *et al.*, 1990, our unpublished data) and mitochondrial presequences have the potential to form amphiphilic α -helices (von Heijne, 1986).

The most conspicuous difference between hydrogenosomal presequences and those of yeast and mammalian mitochondrial proteins is length. The presequences identified thus far for hydrogenosomal proteins are 5–14 amino

acids (Table I), whereas presequences of yeast and mammalian mitochondrial proteins are typically 20–80 amino acids (Hendrick *et al.*, 1989). Nonetheless, presequences as short as 7–12 amino acids are capable of targeting proteins to yeast mitochondria (Hurt *et al.*, 1984; Verner and Lemire, 1989). Interestingly, the length of hydrogenosomal presequences resembles that found on mitochondrial proteins of kinetoplastids, the only protists for which mitochondrial presequences have been examined (Giambiagi-de Marval *et al.*, 1993; Peterson *et al.*, 1993; Searle *et al.*, 1993; Xu and Ray, 1993; Olson *et al.*, 1994). These presequences range in size from 8 to 20 amino acids and function to target proteins to the kinetoplast, an unusual mitochondrion found in the earliest-diverging eukaryotes known to contain mitochondria (Sogin, 1991; Leipe *et al.*, 1993).

Protein import requirements are consistent with a common evolutionary origin for hydrogenosomes and mitochondria

The general parallels between mechanisms used for targeting and translocation of hydrogenosomal and mitochondrial proteins are consistent with recent phylogenetic analyses of hydrogenosomal heat shock proteins (Hsps) that strongly support a common origin for hydrogenosomes and mitochondria. These analyses show that hydrogenosomal Hsps branch within monophyletic groups that otherwise contain exclusively mitochondrial Hsps (Bui *et al.*, 1996; Germot *et al.*, 1996; Horner *et al.*, 1996; Roger *et al.*, 1996; Palmer, 1997). Taking into account the divergence of trichomonads from the main line of eukaryotic descent before the appearance of authentic mitochondria in eukaryotic cells (Sogin, 1991; Leipe *et al.*, 1993), we have proposed that the same endosymbiotic α -proteobacterium gave rise to hydrogenosomes in the anaerobic niches occupied by trichomonads and to mitochondria in the aerobic environment of higher eukaryotes (Bui *et al.*, 1996). As protein import is a trait acquired after the establishment of an endosymbiont with a host cell, the similarities in protein import between hydrogenosomes and mitochondria reported here suggest that the progenitor organelle had evolved prior to the divergence of trichomonads from the main trunk of eukaryotic evolution. As trichomonads are among the earliest-diverging eukaryotes studied to date (Viscogliosi *et al.*, 1993), these data imply that the endosymbiont that gave rise to mitochondria and hydrogenosomes was present in the earliest-diverging eukaryotic lineages. Additional phylogenetic analyses of hydrogenosomal proteins and the protein translocation machinery of hydrogenosomes should help to define the precise evolutionary relationship between hydrogenosomes and mitochondria.

Materials and methods

Parasites

Trichomonas vaginalis C1 (ATCC #30001) was cultured and harvested as described previously (Gorrell *et al.*, 1984).

Isolation of hydrogenosomes

Cells were harvested, lysed and a post-nuclear supernatant was prepared as described previously (Lahti *et al.*, 1992). Hydrogenosomes were then purified using a modification of a procedure used by Opperdoes and colleagues to purify glycosomes (Opperdoes *et al.*, 1984). The post-

nuclear supernatant was subjected to centrifugation at 68 000 g at 4°C for 1 h in 45% Percoll in SMDI [0.25 M sucrose, 10 mM MOPS-KOH (pH 7.2), 10 mM DTT and protease inhibitors TLCK (50 µg/ml) and leupeptin (10 µg/ml)]. Fractions collected from the Percoll gradient were washed twice with 10 volumes of SMDI and re-isolated by centrifugation at 7500 g for 10 min at 4°C. The fractions were resuspended in SMDI and assayed for the presence of hydrogenosomal and lysosomal marker enzymes. Hydrogenosomes were resuspended in freezing buffer [0.25 M sucrose, 10 mM MOPS-KOH (pH 7.2), 3% bovine serum albumin (BSA), 80 mM KCl, 2.5 mM MgCl₂, 20% dimethylsulfoxide (DMSO)] and stored in liquid nitrogen. Before use in *in vitro* import assays, hydrogenosomes were thawed quickly at 37°C, washed twice with 2 volumes of ice-cold SMDI and resuspended in SMDI at a final concentration of 10 mg/ml.

Preparation of cytosolic extract

A 1 liter culture of *T.vaginalis* (~5×10⁹ cells) was pelleted at 1000 g for 10 min at 4°C. Harvested cells were washed twice in SMB [0.25 M sucrose, 10 mM MOPS-KOH, pH 7.2, 10 mM β-mercaptoethanol (β-ME)], once in SMDI, and resuspended to a final concentration of 1.4 g/ml in SMD with 200 µg/ml TLCK and 40 µg/ml leupeptin. Cells were broken by 50 strokes in a 5 ml Teflon dounce on ice, and centrifuged at 10 000 g for 10 min at 4°C. To remove cellular debris and lipid-like material, the supernatant was subjected to three additional centrifugation steps at 16 000 g for 15 min at 4°C in a microcentrifuge.

Enzyme assays

MDH (decarboxylating) was assayed by monitoring the reduction of NADP in the presence of malate at 340 nm as described previously (Steinbüchel and Müller, 1986). Acid phosphatase was assayed using *p*-nitrophenol phosphate as a substrate. Its activity was determined by measuring liberated *p*-nitrophenol at 410 nm as previously described (Müller, 1973). Protein concentrations were determined by the method of Sedmak and Grosberg (1977) or by A₂₈₀ of samples diluted in 0.6% SDS (Yaffe, 1991).

Electron microscopy

Purified hydrogenosomes (100 µg) were cross-linked with 2% glutaraldehyde in SMDI and post-fixed with 1% OsO₄ in phosphate-buffered saline (PBS), dehydrated with ethanol and embedded in Spurr. Approximately 60 µm thick sections were stained with uranyl acetate and lead citrate and examined with a JEOL JEM-100cx electron microscope (JEOL, Ltd, Tokyo, Japan).

Construction of expression plasmids

Constructs encoding full-length pFd, a 'presequence-minus' ferredoxin lacking residues 2–8 (ΔFd) and the precursor ferredoxin with a mutated presequence (FdL2G) were constructed by PCR amplification of the cloned *T.vaginalis* ferredoxin gene (Johnson *et al.*, 1990). Oligonucleotide primers were synthesized and purified on oligonucleotide purification cartridges (Applied Biosystems, Inc.). The pFd 5' primer was a 29mer containing restriction enzyme sites *Pst*I and *Nde*I followed by the first six codons of ferredoxin. The ΔFd 5' primer was a 33mer containing the same restriction enzyme sites followed by a methionine codon and codons 9–14 of ferredoxin. The FdL2G 5' primer contained a *Nde*I site followed by the sequence ATGGGCTCTCAAGTTTGGCGCTTTG, changing leucine at position 2 to glycine. For addition of six C-terminal histidine residues to pFd and ΔFd, the 3' primer was a 52mer complementary to the final six codons of ferredoxin followed by six histidine codons, a termination signal and restriction enzyme sites *Sal*I and *Bam*HI. PCR products were amplified from 10 ng of the cloned ferredoxin gene (Johnson *et al.*, 1990) by 1 U of *Pfu* polymerase in reaction buffer supplied by the manufacturer (Stratagene). PCR cycling temperatures were as follows: five cycles extending 1 min each at temperatures 95, 48 and 72°C followed by 25 cycles extending 1 min each at temperatures 95, 52 and 72°C. The PCR products were digested with *Pst*I and *Bam*HI, cloned into pBS (Stratagene) and sequenced on both strands by the method of Sanger *et al.* (1977) with a Sequenase kit (U.S.Biochemical). Correct clones containing pFd and ΔFd were digested with *Nde*I and *Bam*HI, and the resulting fragments were subcloned into pET3C (Studier and Moffatt, 1986) and used to transform *Escherichia coli* BL21(DE3) cells.

Radiolabeling of proteins

Radiolabeled protein products of pFd and ΔFd were obtained by inducing over-expression and synthesis of these proteins in the presence of radiolabeled amino acids. Five ml of M9 media containing ampicillin

(200 µg/ml) were inoculated with a single colony of *E.coli* BL21(DE3) transformed with pET3C-Fd or pET3C-ΔFd, and grown at 37°C until OD₆₀₀ = 0.5. Cells were pelleted, washed twice and resuspended in 5 ml of M9 salts supplemented with ampicillin (200 µg/ml), 2 mM MgSO₄, 0.2% glucose, 0.1 mM CaCl₂ and 1 mM amino acids (minus cysteine and leucine). The cultures were allowed to grow for 2 h, centrifuged and resuspended in 5 ml of supplemented M9 salts (as above) containing 165 µCi of [³⁵S]cysteine, 8 mCi of [¹⁴C]leucine and 2 mM IPTG. Cells were harvested 2–3 h after induction. pFd and ΔFd were purified on Ni-NTA columns under denaturing conditions using a pH 6.5–4.0 gradient of 8 M urea, 100 mM Na phosphate, 10 mM Tris-HCl for elution as described by the manufacturer (Qiagen). Peak fractions were pooled and dialyzed against 50 mM NaH₂PO₄, 10 mM MgCl₂, 2 mM DDT, 50% glycerol, pH 6.8. Dialyzed samples were concentrated on Millipore MC filters (Millipore). For *in vitro* import experiments with pFdL2G, radiolabeled pFdL2G and pFd proteins were synthesized by *in vitro* transcription followed by translation in a wheat germ cell-free system in the presence of [³⁵S]cysteine according to the instructions of the manufacturer (Promega).

In vitro import assay

Import assays were carried out in a total volume of 100 µl. They included 100 µg of hydrogenosomes, 25 µl of cytosolic extract and 5–20 µl of radiolabeled precursor proteins (~2×10⁵ c.p.m.) in a buffer containing 0.25 M sucrose, 3% BSA, 10 mM MOPS-KOH (pH 7.2), 2.5 mM MgCl₂, 80 mM KCl, 5 mM DTT, 1 mM ATP, 1 mM ADP, 50 µg/ml creatine kinase, 20 mM creatine phosphate, 0.2 mM succinate, 10 mM Na pyruvate, 50 µg/ml TLCK, 10 µg/ml leupeptin. The assay mixture was incubated at 37°C for 30 min, then placed on ice. Organelles were re-isolated by centrifugation, washed twice with ice-cold SMD and resuspended in 0.1 ml of SMD. For the protease protection assay, the sample was divided into thirds. One received an equal volume of SMD, the second received an equal volume of SMD with proteinase K (100 µg/ml final concentration) and the third received an equal volume of SMD with proteinase K (100 µg/ml final concentration) and Triton X-100 (0.5% final concentration). After incubation on ice for 20 min, all samples received phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 2 mM. Samples were mixed with equal volumes of 2× sample buffer [126 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS and 2% β-ME], boiled for 5 min and subjected to 17.5% SDS-PAGE. Gels were fixed, soaked in Autofluor (National Diagnostics), dried and fluorographed for 3–5 days. For import assays in the absence of ATP, the ATP-regenerating system (ATP, ADP, creatine kinase, creatine phosphate, succinate and Na pyruvate) was omitted from the assay. Import reactions were incubated in the presence of 200 U/ml apyrase for 15 min at 37°C before addition of radiolabeled precursor protein. Neither the presence or absence of the C-terminal six histidine residues nor synthesis *in vivo* or *in vitro* affected import of pFd (data not shown).

CCCP treatment

For import assays in the presence of CCCP, cytosol was prepared in the absence of DTT and DTT was omitted from the import buffer. CCCP was added from a 40-fold concentrated stock solution in ethanol and pre-incubated with hydrogenosomes in import buffer for 5 min at 37°C before the addition of radiolabeled pFd. The samples were made chemically identical by adding the same amount of reagent-free solvent to the control samples.

NEM and heat inactivation of T.vaginalis cytosol

Cytosol for NEM experiments was prepared in the absence of DTT. The cytosol was treated with 10 mM NEM for 15 min at room temperature, followed by quenching the NEM with 20 mM DTT. Controls received 20 mM DTT just prior to 10 mM NEM addition. The treated cytosol was then added immediately to import reactions. Heat inactivation of the cytosol was performed at 90°C for 10 min. The extract was then centrifuged for 5 min at 14 000 g to remove precipitated proteins, and the supernatant was added immediately to import reactions.

Acknowledgements

We thank Guadalupe Delgadillo for excellent technical assistance and Drs Miklos Müller, Greg Payne, Dan Ray and Alexander van der Blik and members of our laboratory for helpful discussions and critical review of the manuscript. Electron microscopy was carried out by the Biomedical E.M. Core Facility, UCLA. This work was supported by NIH grant AI27857 to P.J.J., a predoctoral training grant (NIH AI07323) to P.J.B.,

a postdoctoral fellowship (PI 218/1-1) from the Deutsche Forschungsgemeinschaft to E.P. and a Burroughs Wellcome New Investigator in Molecular Parasitology Award to P.J.J.

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Received on October 28, 1996; revised on March 5, 1997