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Hypothesis

Import of a mitochondrial presequence into *P. denitrificans*

Insight into the evolution of protein transport

David Roise*, Merritt Maduke

Department of Chemistry, University of California at San Diego, La Jolla, CA 92093–0506, USA

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Abstract

According to the endosymbiont hypothesis, mitochondria are descended from ancient aerobic bacteria that were engulfed by protoeukaryotic cells. Experiments described here show that a synthetic peptide corresponding to a yeast mitochondrial targeting sequence can be imported into *Paracoccus denitrificans*, a soil bacterium thought to be closely related to the protomitochondrion. The import is very similar to that observed with isolated yeast mitochondria. The results suggest that the protomitochondrion may have been inherently able to translocate mitochondrial presequences. This ability may partly explain the development of the protein import process during the evolution of the mitochondrion.

Key words: Bacterial membrane; Evolution; Mitochondrion; Protein translocation

1. Introduction

The evolution of modern mitochondria began 1 to 1.5 billion years ago, when primitive aerobic bacteria were engulfed by nascent eukaryotic cells [1,2]. Over time, most of the genes carried by the protomitochondria were copied into the nucleus of the eukaryotic cells. In parallel with the copying process, the proteins encoded by the transferred genes acquired amino-terminal extensions that were responsible for the subsequent targeting of the nuclear-encoded proteins into the evolving organelle. Once the mitochondria had established the ability to import the nuclear-encoded proteins, the mitochondrial copies of the genes coding for these proteins could be eliminated without loss of mitochondrial function. Today, only a handful of mitochondrial proteins remain encoded by the mitochondrial genome.

Although the origin and evolutionary history of the protein transport system in mitochondria is unknown, the amino-terminal targeting sequences (presequences) obtained by the nuclear-encoded mitochondrial proteins during the copying process were probably responsible for engaging whatever primitive transport system was present in the early mitochondria. Modern mitochondrial presequences display common structural and physical properties but lack conserved primary sequences [3]. Presequences normally function independently of the proteins to which they are attached and are typically removed by proteolysis after they have reached the interior of a mitochondrion [4,5]. Presequences are typically positively-charged, and their translocation into the matrix space depends on the electrical potential across the inner mitochondrial membrane [6,7].

The ability of protomitochondria to recognize and translocate presequences was probably a critical requirement for the development of a protein transport system by the primitive organelle. In the work described here, we show that *Paracoccus denitrificans*, a soil bacterium that is believed to be closely related to the protomitochondrion, can bind and import a synthetic mitochondrial presequence. The import depends on the electrical potential of the plasma membrane and was not observed with a mutated presequence known to be less efficient in importing attached proteins into mitochondria. The results show that these bacteria have the inherent ability to recognize and translocate a mitochondrial targeting sequence and suggest a possible mechanism for the devel-

*Corresponding author. Fax: (1) (619) 534-4864.

Abbreviations: CoxIV, cytochrome oxidase subunit IV; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone; HPLC, high pressure liquid chromatography; NBD, 7-nitro-2,1,3-benzoxadiazolyl; Δ11,12, presequence lacking residues 11 and 12 of the yeast CoxIV precursor.

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opment of a protein import machinery in primitive mitochondria.

2. Materials and methods

Cultures of *P. denitrificans* (ATCC 13543) were grown on succinate medium [8] to mid-log phase (A_{600} = 0.2) at 30°C. The cells were washed once with medium and resuspended in 1/500th the original volume of fresh medium. Concentrations were determined by drying aliquots of the cells under reduced pressure. Yeast mitochondria were isolated from *Saccharomyces cerevisiae* (D273-10B) as described [9]. The synthetic presequence, Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Phe-Lys-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser-Ser-Arg-Tyr-Leu-Leu-NH_2, corresponds to the amino-terminal 25 residues of the yeast cytochrome oxidase subunit IV (CoxIV) prescursor protein. It was labeled at its single cysteine with 7-nitro-2,1,3-benzoxadiazolyl (NBD) and purified as described [10]. Binding and translocation experiments with cells and with mitochondria were performed essentially as reported previously with lipid vesicles [10]. Solutions contained 10 mM KP_i, pH 7.2, 50 mM KCl, 1 mM $MgCl_2$, and 1 mM 1,10-phenanthroline (standard buffer) at 20°C. Mitochondrial solutions also contained 0.6 M D-sorbitol. The concentration of presequence was 100 nM for all experiments. The presequence was added from a 20 μM stock solution in 50% EtOH. Fluorescence was measured with an SLM-Aminco SPF-500C spectrofluorometer (excitation 485 nm; emission 540 nm; 5 nm bandpass).

3. Results and discussion

In order to test whether modern aerobic bacteria might be able to recognize and translocate mitochondrial presequences, we have used a synthetic peptide corresponding to the amino-terminal 25 residues of the yeast CoxIV precursor protein. The physical properties of this presequence as a cationic, amphiphilic helix have been described extensively [11–15]. The presequence has been covalently modified with fluorescent probes at its single cysteine residue, so that interactions with membranes can be monitored spectroscopically. The labeled presequence is rapidly and efficiently imported into isolated yeast mitochondria and also into phospholipid vesicles [10,16]. In both cases, the import depends on a transmembrane electrical potential.

Since the CoxIV presequence can be translocated in a potential-dependent manner into phospholipid vesicles, we speculated that bacteria, which express membrane potentials of similar magnitude and polarity to those observed in mitochondria, might also be able to translocate presequences. This ability could suggest a mechanism for the development of a protein import pathway in protomitochondria. When *E. coli* were tested, however, no binding or import of the presequence was observed (S.T. Swanson and R. Davis, unpublished), perhaps because of the hydrophilic barrier of the bacterial outer membrane [17]. In contrast, another Gram-negative bacterial strain, *Paracoccus denitrificans*, proved much more interesting in our assays. These soil bacteria are thought to be closely related to modern mitochondria [18].

Binding of the fluorescent CoxIV presequence to *P. denitrificans* cells was measured by the enhancement of fluorescence of the NBD label in the bound state (Fig. 1A). The binding curve is described by a hyperbolic function as expected for a two-state equilibrium between the free and bound forms of the presequence. The bound form represents presequences that have partitioned non-specifically into an accessible lipid bilayer [16,19]. A similar binding curve was obtained with isolated yeast mitochondria (Fig. 1B), although the units of concentration are not directly comparable.

Translocation of a presequence across membranes protects the presequence from digestion by added pro-

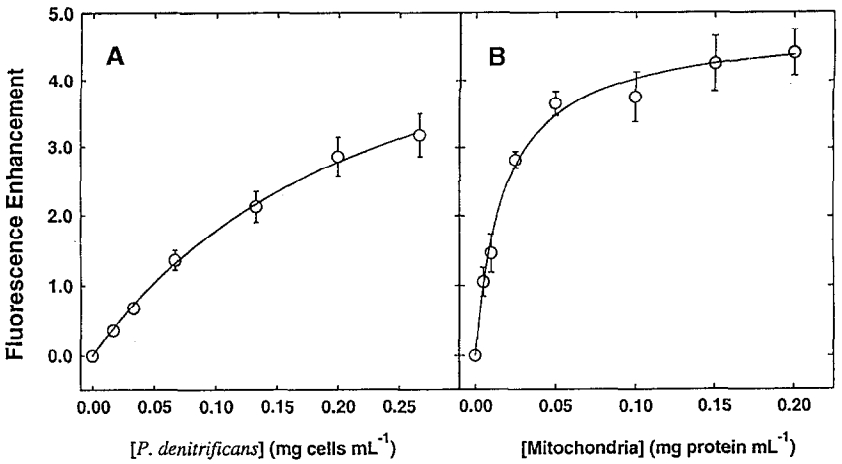

Fig. 1. Binding of the NBD-labeled CoxIV presequence to *P. denitrificans* cells (A) and to isolated yeast mitochondria (B). Fluorescence enhancement is defined as: $(F - F_o)F_o^{-1}$, where F is the observed fluorescence of the presequence in the presence of cells or mitochondria, and F_o is the fluorescence in the absence of cells or mitochondria. Data were fitted to: $(F - F_o)F_o^{-1} = [(F_{max} - F_o)\,F_o^{-1}][M]([M] + M_{50})^{-1}$, where [M] is the concentration of cells or mitochondria. F_{max} and M_{50} are constants that correspond respectively to the fluorescence of the fully bound presequence and to the concentration of cells or mitochondria where half-maximal enhancement is observed. Best fits of the data gave values of $(F_{max} - F_o)F_o^{-1}$ = 6.3 and M_{50} = 0.25 mg cells · ml^{-1} for *P. denitrificans*, and $(F_{max}-F_o)F_o^{-1}$ = 4.8 and M_{50} = 0.019 mg protein · ml^{-1} for mitochondria. The lines shown were generated from these values using the equation above.

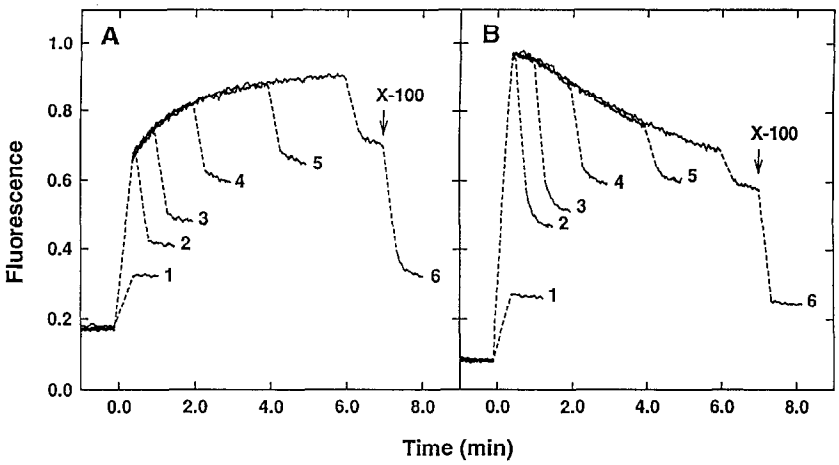

Fig. 2. Translocation of the presequence into *P. denitrificans* (A) and into mitochondria (B). In each case, six separate experiments are superimposed. *P. denitrificans* cells (0.2 mg) or mitochondria (0.05 mg) were added initially to standard buffer (1 ml) in a fluorescence cuvette. The initial signal is due to scattered light in these samples. At time 0, the presequence was added. Trypsin (0.05 mg) either was present at the beginning of the experiment (trace 1) or was added at 0.5 min, 1 min, 2 min, 4 min, or 6 min (traces 2–6). Addition of Triton X-100 (0.01%) and EDTA (2 mM) at the end of an experiment (X-100) released the protected presequence and returned the fluorescence to that of the free fluorophore (trace 6).

teases [10,16]. The protection can be monitored by adding protease at various times after addition of the presequence and measuring changes in the fluorescence as the untranslocated presequence is digested by the protease and released from the surface of the membrane. From the changes in fluorescence, the amount of presequence protected at each time can be calculated [16]. Experiments with *P. denitrificans* showed a time-dependent protection of the presequence from added trypsin (Fig. 2A). This result suggests that the presequence was translocated into the cells. The fluorescence observed immediately after the addition of the presequence to the cells was relatively low, because the concentration of cells used was less than the value of M_{50}; less than half of the total presequence was initially bound. Higher concentrations of cells could not be used in these experiments because of light-scattering artifacts. The time-dependent increase in fluorescence observed prior to the addition of trypsin suggests that the presequence remains associated with the inner surface of the plasma membrane following its translocation and retains an enhanced fluorescence. The total fluorescence increases because additional presequences associate with the exterior surface to maintain the binding equilibrium. Of the 100 nM total initial concentration of the presequence, 12, 21, 32, 33, and 40 nM were protected at 0.5, 1.0, 2.0, 4.0, and 6.0 min, respectively.

Analogous experiments with yeast mitochondria

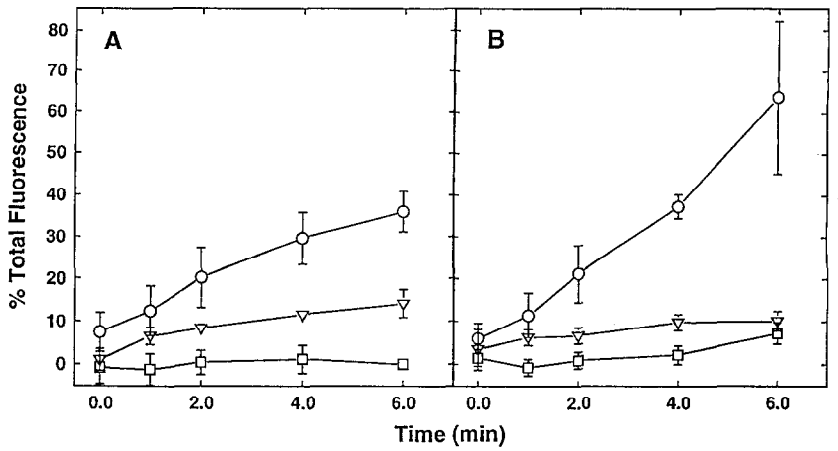

Fig. 3. Association of the protected presequence with the *P. denitrificans* cells (A) and with mitochondria (B). Conditions were the same as in Fig. 2, except that the samples were treated with soy bean trypsin inhibitor (0.1 mg) after digestion by trypsin for 1.5 min on ice. The cells and mitochondria were then re-isolated by centrifugation (14,000 × *g*, 4°C, 2 min for *P. denitrificans* and 5 min for mitochondria). The resulting pellets were resuspended in methanol (1 ml) using an ultrasonic bath and the fluorescence was measured. Values are corrected for background scattering and are plotted relative to the total possible fluorescence. Standard import (○); samples treated with FCCP (5 μM) and gramicidin (2.5 $\mu g \cdot ml^{-1}$) (▽); import measured with the NBD-labeled Δ11,12 mutant presequence (□).

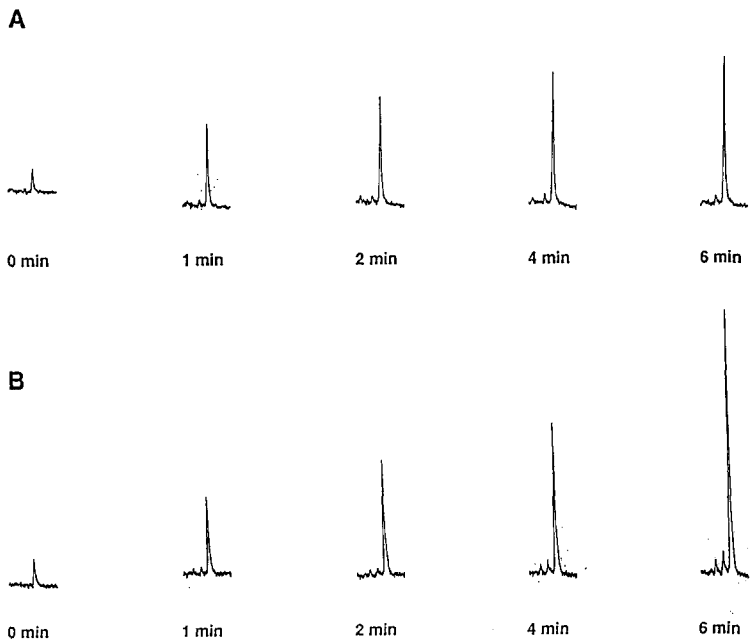

Fig. 4. Analysis of the protected presequence by HPLC. Samples obtained from the experiments described in Fig. 3 were dried and re-dissolved in 0.2% trifluoroacetic acid, 25% acetonitrile. They were chromatographed by reverse-phase HPLC (Vydac C-18) with continuous monitoring of the fluorescence (Fluoromonitor III, Laboratory Data Control Inc.; excitation 440 nm; emission 500–700 nm). Under standard import conditions (+1,10-phenanthroline), the major fluorescent peak corresponded to the intact presequence. These peaks are shown for each time point of import with *P. denitrificans* cells (A) and with mitochondria (B).

showed similar results (Fig. 2B) [16]. With mitochondria, however, the fluorescence observed upon addition of the presequence was initially higher than that with *P. denitrificans*, because the concentration of mitochondria used was larger than the value of M_{50}; under these conditions, roughly 80% of the total presequence was in the bound form. The decrease in fluorescence during the experiment suggests that the fluorescence of presequences is quenched inside the mitochondria. From the changes in fluorescence observed upon addition of trypsin, it was found that 30, 36, 60, 78, and 83 nM were protected at 0.5, 1.0, 2.0, 4.0, and 6.0 min, respectively. Thus, a substantial fraction of the total presequence became protected from digestion by protease with both the bacterial cells and with mitochondria. In all cases, the protection could be reversed upon disruption of the bacterial and mitochondrial membranes by treatment with Triton X-100 and ethylenediaminetetraacetic acid (EDTA).

The protected presequence remained tightly associated with the *P. denitrificans* cells and with the mitochondria (Fig. 3). Centrifugation of the samples following treatment with trypsin showed a time-dependent increase in the amount of fluorescence associated with the pellets. Consistent with the direct fluorescence measurements (Fig. 2), up to 35% of the labeled presequence was recovered with the *P. denitrificans* cell pellet after treatment with trypsin at 6 min. For mitochondria, roughly 60% of the labeled presequence was recovered at the same time. In each case, the protection could be significantly decreased by dissipation of the membrane potential with carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and gramicidin. Since the membrane potential is expressed across the plasma membrane of the *P. denitrificans* cells and across the inner membrane of the mitochondria, this result suggests that the protection corresponds to translocation of the presequence across these membranes. As a control, a mutant form of the CoxIV presequence that lacks two residues at positions 11 and 12 (Δ11,12) was found to be protected much less efficiently with both *P. denitrificans* and mitochondria. This result is consistent with the decreased effectiveness of this sequence in importing attached proteins into mitochondria in vitro and in vivo [20,21]. It shows that changes in the primary structure of a presequence alter the transport of the presequence into *P. denitrificans* and into mitochondria in a similar manner.

The presequence is digested by metal-dependent peptidases within both the *P. denitrificans* cells and within the mitochondria. Solutions typically contained 1,10-phenanthroline, a membrane-permeant metal chelator, to minimize this effect. In the presence of the chelator, the presequence was found by high pressure liquid chromatography (HPLC) to remain intact following its import into both *P. denitrificans* and mitochondria (Fig. 4). In the absence of the chelator, however, less of the intact presequence was recovered by HPLC, and new, less hydrophobic, fragments were observed.

The experiments reported here demonstrate that a mi-

tochondrial presequence can be imported into *P. denitrificans* cells in a potential-dependent manner, and that this process displays striking similarities to that observed with isolated mitochondria. The results give additional support for the suggestion that *P. denitrificans* serves as an especially good model for the ancestral form of the modern mitochondrion [18]. Although it is not clear from these experiments whether the presequence passes through the lipid phase, through a protein channel, or through some combination of the two as it is imported into the cells, two unique features of *P. denitrificans* may be important for facilitating uptake of the presequence. First, the cells are unusually sensitive to hydrophobic agents, possibly due to an unusual outer membrane architecture [22,23]. This sensitivity may explain the facile binding of the presequence to the bacterial surface (Fig. 1). Second, unlike most other bacteria, *P. denitrificans* contains phosphatidylcholine rather than phosphatidylethanolamine as the major zwitterionic lipid [24]. We have found that phosphatidylethanolamine greatly decreases the ability of the CoxIV presequence to be translocated across bilayers in which it is present (M. Maduke, S.T. Swanson, R. Davis and D. Roise, unpublished).

The results provide support for a relatively simple series of events leading to the development of the protein import process in mitochondria. Several studies have shown that random segments of DNA can encode large numbers of functional presequences [25,26]. Attachment of these sequences, which are always cationic and which are predicted to be amphiphilic, to mitochondrial proteins by gene fusion could have generated mitochondrial precursor proteins as the mitochondrial genes were copied into the nucleus. If the protomitochondrion could inherently recognize and translocate presequences, as the results presented here suggest, the precursor proteins generated by gene fusion in the nucleus could at least be initiated into the import pathway following their synthesis in the cytoplasm. Optimization of the translocation process by the mobilization and evolution of components within the mitochondrial membranes could then continue as the organelle developed. The import of full-length precursor proteins into *P. denitrificans* has not yet been tested, but the ability of the targeting portion of a precursor protein to be imported by these bacteria provides intriguing clues about the evolution of this critical process in eukaryotic cells.

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References

[1] Margulis, L. (1981) in: Symbiosis in Cell Evolution, W.H. Freeman and Co., San Francisco.
[2] Gray, M.W. (1989) Trends Genet. 5, 294–299.
[3] Roise, D. and Schatz, G. (1988) J. Biol. Chem. 263, 4509–4511.
[4] Hurt, E.C. and Van Loon, A.P.G.M. (1986) Trends Biochem. Sci. 11, 204–207.
[5] Hartl, F.U., Pfanner, N., Nicholson, D.W. and Neupert, W. (1989) Biochim. Biophys. Acta 988, 1–45.
[6] Schleyer, M. and Neupert, W. (1985) Cell 43, 339–350.
[7] Pfanner, N. and Neupert, W. (1985) EMBO J. 4, 2819–2825.
[8] Scholes, P. and Mitchell, P. (1970) J. Bioenerg. 1, 61–72.
[9] Daum, G., Böhni, P.C. and Schatz, G. (1982) J. Biol. Chem. 257, 13028–13033.
[10] Maduke, M. and Roise, D. (1993) Science 260, 364–367.
[11] Roise, D., Horvath, S.J., Tomich, J.M., Richards, J.H. and Schatz, G. (1986) EMBO J. 5, 1327–1334.
[12] Tamm, L.K. (1986) Biochemistry 25, 7470–7476.
[13] Endo, T., Shimada, I., Roise, D. and Inagaki, F. (1989) J. Biochem. (Tokyo) 106, 396–400.
[14] Tamm, L.K. and Bartoldus, I. (1990) FEBS Lett. 272, 29–33.
[15] Frey, S. and Tamm, L.K. (1990) Biochem. J. 272, 713–719.
[16] Roise, D. (1992) Proc. Natl. Acad. Sci. USA 89, 608–612.
[17] Nikaido, H. and Vaara, M. (1985) Microbiol. Rev. 49, 1–32.
[18] John, P. and Whatley, F.R. (1975) Nature 254, 495–498.
[19] Swanson, S.T. and Roise, D. (1992) Biochemistry 31, 5746–5751.
[20] Allison, D.S. and Schatz, G. (1986) Proc. Natl. Acad. Sci. USA 83, 9011–9015.
[21] Roise, D., Theiler, F., Horvath, S.J., Tomich, J.M., Richards, J.H., Allison, D.S. and Schatz, G. (1988) EMBO J. 7, 649–653.
[22] Wilkinson, B.J. (1977) FEMS Microbiol. Lett. 2, 285–288.
[23] Nikaido, H. (1976) Biochim. Biophys. Acta 433, 118–132.
[24] Wilkinson, B.J., Morman, M.R. and White, D.C. (1972) J. Bacteriol. 112, 1288–1294.
[25] Baker, A. and Schatz, G. (1987) Proc. Natl. Acad. Sci. USA 84, 3117–3121.
[26] Lemire, B.D., Fankhauser, C., Baker, A. and Schatz, G. (1989) J. Biol. Chem. 264, 20206–20215.