The N-terminal 21 amino acids of a 70 kDa protein of the yeast mitochondrial outer membrane direct *E. coli* β-galactosidase into the mitochondrial matrix space in yeast cells

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The intracellular location of fusion proteins was investigated in yeast cells. They consisted of the N-terminal 21, 61 or 292 amino acids of the 70 kDa protein of the yeast mitochondrial outer membrane and an enzymatically active *E. coli* β-galactosidase. The hybrids containing 61 or 292 residues of the 70 kDa protein, as well as the original 70 kDa protein, were localized on the outer membrane in a tightly membrane-bound form. In contrast, the other hybrid was exclusively localized in the mitochondrial matrix space as a soluble protein.

Mitochondrial protein import *(Yeast)* *Mitochondrial outer membrane* *Fusion protein* *Membrane protein* *Membrane binding*

1. INTRODUCTION

Virtually all mitochondrial proteins are coded by nuclear genes and synthesized in the extramitochondrial cytoplasm. They are then transported to their correct compartments in the mitochondrion; this transport plays a centrole role in the biogenesis of this organelle [1,2]. The transport process of the mitochondrial outer membrane proteins to their final destination is at least partly different from those for internal mitochondrial proteins [3–6] and involves at least the following two steps: (i) the targeting step, a specific interaction and binding of the proteins to the cytoplasmic surface of the mitochondria, probably involving a receptor; and (ii) the anchoring step, an energy-independent insertion and permanent association of the binding molecule to the outer membrane. A major 70 kDa protein, like other proteins belonging to the yeast mitochondrial outer membrane, is synthesized without the N-terminal extension [5] and all the information necessary for localizing this protein on the outer membrane is contained within the N-terminal 41 amino acids [7].

This paper shows definite evidence that the first 21 amino acids of the 70 kDa protein are sufficient for the targeting to the mitochondria but not for the anchoring to the outer membrane.

Abbreviations: N-, amino-; ONPG, *O*-nitrophenyl galactoside

2. EXPERIMENTAL

Fusion genes of the 70 kDa protein and β-galactosidase were constructed as in [7] and the resulting proteins coded by the fusion genes contain respectively 21, 61, and 292 amino acids of the 70 kDa protein at the N-terminus and a constant acids of the 70 kDa protein at the N-terminus and a constant portion of β-galactosidase at the carboxy-terminus, and are referred to as (1–21)LacZ′, (1–61)LacZ′, and (1–292)LacZ′

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proteins, respectively. Yeast strain, growth of cells, and various cell fractionations were as described in [7] except that yeast cells were grown for 12 h in a rich semi-synthetic medium [8]. β-Galactosidase activity was assayed using ONPG as a substrate according to Miller [9]. Lactose-hydrolyzing activity of β-galactosidase in mitochondria was determined as follows. Mitochondria (300 μg) were incubated in 250 μl composed of 0.6 M sorbitol, 20 mM Tris-HCl (pH 7.4), 10 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, and 50 mM lactose for 30 min at 27°C in the absence or presence of 0.2% Triton X-100, and then heated for 5 min at 100°C. The amount of glucose produced by hydrolysis of lactose was determined as in [10]. Activities of marker enzymes, cytochrome b_2 [11], cytochrome-*c* oxidase [12], and malate dehydrogenase [13] were measured spectrophotometrically with slight modifications of the original procedures. In some cases, membrane fractions were pretreated with 0.1% Triton X-100 to abolish enzyme latency.

3. RESULTS

3.1. *Subcellular distribution of 70 kDa-LacZ' fusion proteins*

The subcellular distribution of the fusion proteins was determined by fractionation of yeast cells into mitochondria, microsomes, and cytosol fractions (table 1). The original β-galactosidase remained in the cytosol, whereas all fusion proteins were exclusively co-fractionated with mitochondria. In the previous experiment [7], a major part of the (1-21)LacZ' protein was recovered in the cytosol fraction in contrast to the present result.

Table 1

Subcellular distribution of the 70 kDa-LacZ' fusion proteins

Fusion proteins	Distribution (%)		
	Mitochondria	Microsomes	Cytosol
(1-21)LacZ'	76	9	15
(1-61)LacZ'	87	8	5
(1-292)LacZ'	86	10	4
Original β-galactosidase	3	5	92

Although this discrepancy cannot be explained at the moment, the observation of the mitochondrial localization of (1-21)LacZ' protein was reproducible.

3.2. *Protease accessibility of the fusion proteins in intact mitochondria*

The 70 kDa protein is anchored to the mitochondrial outer membrane via its N-terminal region and

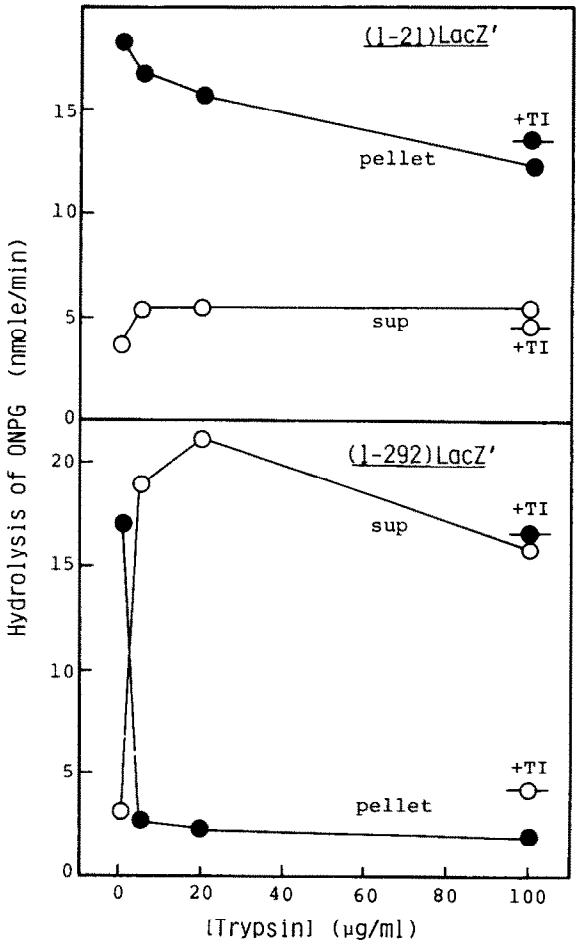

Fig.1. Differences in accessibility of 70 kDa-LacZ' fusion proteins in intact mitochondria to exogenous trypsin. Mitochondria (1 mg/ml) containing (1-21)LacZ' or (1-292)LacZ' protein were treated with 0, 5, 20 or 100 μg/ml of trypsin for 15 min at 0°C, after which soybean trypsin inhibitor (TI) was added to 500 μg/ml. As a control, mitochondria were treated with trypsin (100 μg/ml) in the presence of TI. Mitochondria were recovered by centrifugation at 10000 × *g* for 5 min. β-Galactosidase activity was measured in the resulting supernatants (○—○) and trypsinized mitochondria (●—●).

most of the molecule (60 kDa) is exposed to the cytosolic side [7,14]. If the fusion proteins were inserted into the outer membrane topographically in a similar manner to the original 70 kDa protein, the β-galactosidase part would be exposed on the mitochondrial surface. When the mitochondria were incubated with exogenous trypsin, which attacks only the surface of mitochondria, the β-galactosidase activity of (1-292)LacZ′ protein was easily released without any significant loss of enzymic activity, suggesting that the activity itself was trypsin-resistant, whereas that of (1-21)LacZ′ protein was retained in the mitochondria (fig.1). (1-61)LacZ′ protein was also attacked by exogenous trypsin (not shown). These results show that (1-292)LacZ′ and (1-61)LacZ′ proteins are attached to the mitochondrial surface and that (1-21)LacZ′ protein is located inside the mitochondrion.

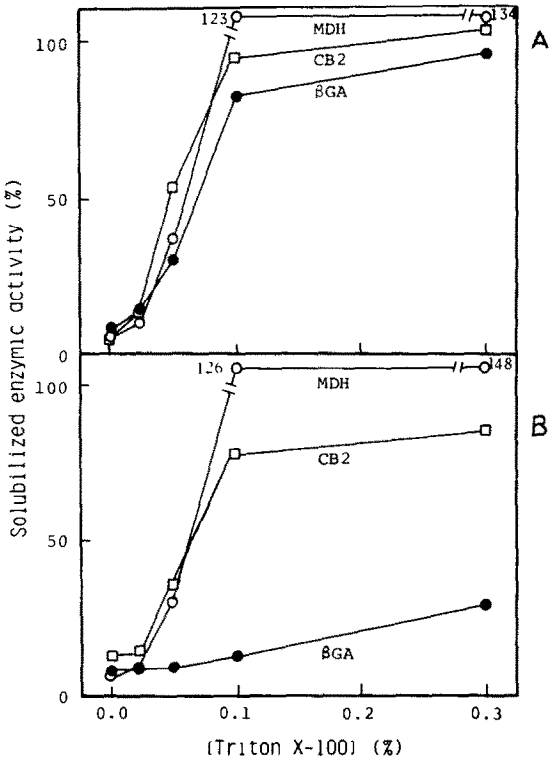

Fig.2. Solubilization of 70 kDa-LacZ′ proteins from mitochondria by Triton X-100 treatment. Mitochondria containing (1-21)LacZ′ (A) or (1-292)LacZ′ (B) protein (5 mg/ml) were incubated with the indicated amount of Triton X-100 for 5 min at 0°C and solubilized proteins were obtained by centrifugation at 100 000 × g for 1 h. The supernatants were assayed for activities of β-galactosidase (●—●), malate dehydrogenase (○—○), and cytochrome b_2 (□—□). The results are plotted as percentages of the activities found in the original mitochondria.

3.3. *Submitochondrial location of the fusion proteins*

When the mitochondria were severely disrupted by sonication or treated with Triton X-100, (1-21)LacZ′ protein was easily liberated into the supernatant in parallel with cytochrome b_2 and malate dehydrogenase, an intermembrane space and a matrix protein, respectively (figs 2A and 3A), whereas (1-292)LacZ′ protein was retained in the membrane fraction as tightly as a typical mem-

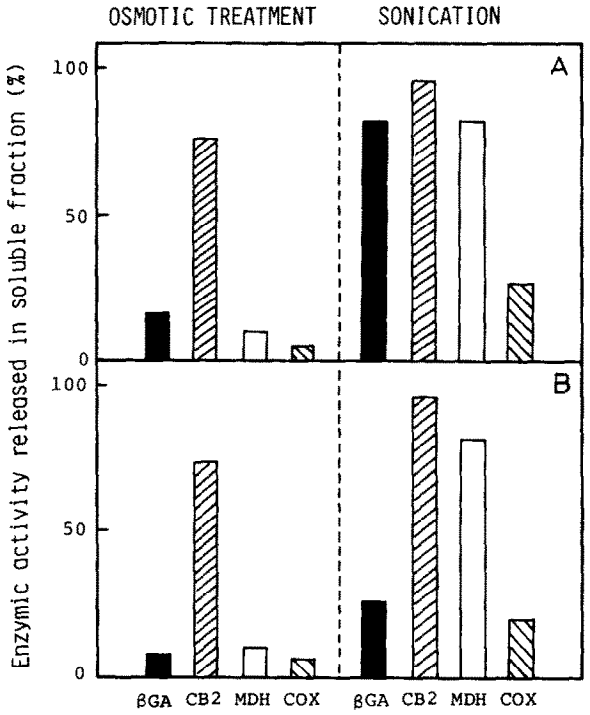

Fig.3. Effects of osmotic treatment and sonication on the association of 70 kDa-LacZ′ fusion proteins with mitochondria. Mitochondria containing (1-21)LacZ′ (A) or (1-292)LacZ′ protein (B) were ruptured by osmotic treatment or sonication and reisolated by centrifugation. The supernatants were assayed for activities of β-galactosidase (βGA), cytochrome b_2 (CB2), malate dehydrogenase (MDH), and cytochrome-*c* oxidase (COX). The activities are expressed as percentages of those found in the original mitochondria.

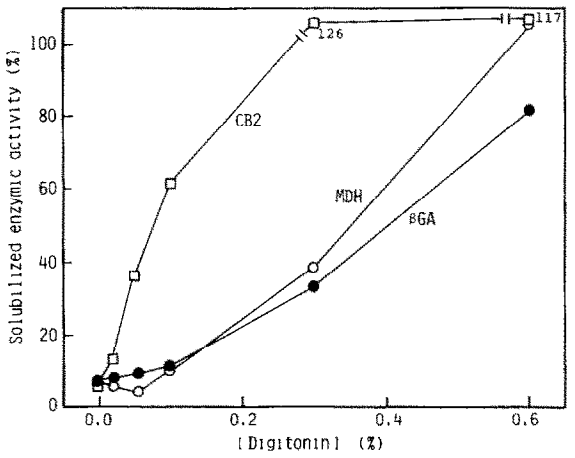

Fig.4. Differential solubilization of the outer and inner membranes of mitochondria containing (1-21)LacZ′ protein by digitonin treatment. Mitochondria (5 mg/ml) were incubated with the indicated amounts of digitonin for 5 min at 0°C and solubilized proteins obtained and assayed as described in fig.2.

brane protein, cytochrome-*c* oxidase (figs 2B and 3B). (1-61)LacZ′ protein showed similar behavior to (1-292)LacZ′ protein (not shown). By combining these observations and the trypsin accessibility, (1-292)LacZ′ and (1-61)LacZ′ proteins behaved essentially as outer membrane proteins, probably anchored via the 70 kDa protein moiety.

Upon preferential disruption of the outer membrane by treatment of the mitochondria with a hypotonic solution or low concentration of digitonin, (1-21)LacZ′ protein was not released from mitochondria (figs 3A and 4). By increasing the concentration of digitonin, which leads to further release of matrix proteins, (1-21)LacZ′ protein was recovered in the supernatant in parallel with malate dehydrogenase (fig.4). These data suggest that (1-21)LacZ′ protein is located in the mitochondrial matrix space as a soluble form.

3.4. *Latency of β-galactosidase activity*

The β-galactosidase activity was measured using lactose as a substrate which freely penetrates the outer, but not the inner membrane. If β-galactosidase were located inside the inner membrane, the lactose-hydrolyzing activity would be masked. As shown in table 2, the activity of (1-21)LacZ′ protein was significantly lower in the intact mitochondria and an approx. 5-fold stimulation was observed after disruption of membranes with Triton X-100. In contrast, full activities of (1-292)LacZ′ and (1-61)LacZ′ proteins were obtained in the intact mitochondria. These data indicate that the activity itself is not influenced by Triton X-100 and therefore, the lower activity of (1-21)LacZ′ protein must reflect the location of this protein in the matrix space.

4. DISCUSSION

This study has demonstrated clearly that β-galactosidase whose N-terminus is joined to the N-terminal 21 residues of the 70 kDa protein is localized on the mitochondrial matrix in living cells. This result provides strong evidence that the targeting step of the 70 kDa protein is mediated by the region composed of at most the first 21 residues. Permanent association of this protein to the outer membrane requires subsequent residues,

Table 2

Effect of Triton X-100 on the lactose-hydrolyzing activity of the 70 kDa-LacZ′ fusion proteins in mitochondria

Fusion proteins	Assay condition		Stimulation by Triton X-100 (-fold)
	−Triton X-100	+Triton X-100	
(1-21)LacZ′	1.48 ± 0.12	7.03 ± 0.20	4.25
(1-61)LacZ′	5.78 ± 0.47	6.23 ± 0.56	1.08
(1-292)LacZ′	4.56 ± 0.05	4.90 ± 0.36	1.07

Enzymic activity was assayed using 300 μg mitochondria in the absence or presence of 0.2% Triton X-100 as described in section 2. The amounts of glucose (μmol) produced/30 min per mg mitochondrial protein are expressed as an average of 3 measurements ± SD

with which a typical transmembrane structure is achieved: a stretch of uncharged amino acids running from 10 to 37 flanked on both sides by basic residues as determined previously [15]. This has also been confirmed by the fact that (1-61)LacZ′ and (1-292)LacZ′ are properly localized on the outer membrane. The N-terminal sequence of (1-21)LacZ′ protein including the junction region resembles that of the original 70 kDa protein in terms of both the net charge and the position of the charged residues: a long uninterrupted stretch of 24 uncharged residues with clusters of basic residues on both sides

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 + +     + +
(MKSFITRNKTAILATVAATGTG-
                + + +   +
  SGAWLLPVSLVKRKTTLR;
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the region derived from the 70 kDa protein is underlined). In spite of this similarity, the above sequence could not serve as a membrane anchor or stop transfer sequence. This suggests that a specific sequence is necessary to stop protein translocation across the mitochondrial membranes. Recently, yeast porin, a major mitochondrial outer membrane protein, has been shown to have an N-terminal sequence similar to the targeting sequence of the 70 kDa protein [16], suggesting the possibility that such a homologous region functions as a common targeting signal for outer membrane proteins.

An important point that emerges from this work is the demonstration that the targeting sequence of the outer membrane protein can mediate the translocation of an attached polypeptide across the mitochondrial membranes. This implies that neither the signal(s) for translocation in imported proteins nor the translocation apparatus of mitochondria is specific for internal mitochondrial proteins.

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