MULTIPLICITY OF MITOCHONDRIAL PROTEINASES IN YEAST

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Received 13 October 1981

1. Introduction

Several mitochondrial proteins made both on cytoplasmic and mitochondrial ribosomes have been reported to be initially synthesized as larger precursors. Transport of such precursors across mitochondrial membranes and their insertion into membranes is believed to be associated with their post-translational [1] or co-translational [2] processing to mature forms by mitochondrial proteolytic enyzmes.

However, it has been found [3–5] that the mitochondrial inner membrane possesses a proteinase rapidly digesting mitochondrial translation products which probably play an organizing role in mitochondrial assembly. Thus it is important to gain information on mitochondrial proteinases: their number, functions and regulation of activity.

The first attempt to isolate a mitochondrial proteinase was made in Bartley's laboratory [6] but was not followed up. The quest was later taken up by two groups of investigators: Jusič et al. [7] have isolated a proteinase cleaving histones from rat-liver mitochondria; a proteinase performing limited proteolysis of apoproteins of some pyridoxal-dependent enzymes was obtained from the same source by Katunuma et al. [8]. Subsequently there was a report [9] on the possible identity of these enzymes.

These lines of research were, however, nonplussed by the finding [10] that the proteolytic activity of rat-liver mitochondria is in all probability associated with mast cell granules co-sedimenting with mitochondria during isolation from liver tissue. Thus, liver appeared to be unfit for isolation of and studies on mitochondrial proteinases, as are other organs comprizing different cell types.

This problem does not arise in works with eukaryotic microorganisms. According to [11], the matrix of *Saccharomyces cerevisiae* mitochondria harbours a proteinase processing the precursors to the 3 largest subunits of F_1-ATPase and subunit V of cytochrome oxidase. However, the processor proteinase has not been isolated. Furthermore, it remains obscure which and how many proteinases are localized in the mitochondrial inner membrane where the co-translational processing (see, e.g., [2]) and breakdown [4] of mitochondrial translation products take place. Taking into account the multiplicity of functions of mitochondrial proteinases, it is reasonable to expect the multiplicity of physical entities carrying out these functions.

This work presents an experimental approach to assessing the number of proteinases associated with the yeast mitochondrial inner membrane.

2. Materials and methods

The strain of *S. cerevisiae* and growth conditions used have been described [12]. Submitochondrial particles (SMP) were prepared as in [13] from mitochondria isolated as in [14]. Cytochrome *c* was labelled with [^{3}H]borohydride according to [15]. The specific radioactivity of the resultant preparation was 90 μCi/mg or 1.1 Ci/mmol. Cytochrome *c* hydrolase was assayed by incubating 0.25 mg SMP protein with 11.25 μg [^{3}H]cytochrome *c* in a final volume of 0.2 ml of 30 mM Tris–HCl (pH 7.4), 0.2 mM ethylene diamine tetraacetic acid. After 60 min at 37°C, the reaction was terminated by successive additions of 100 μl of the above buffer, 100 μl of albumin solution (0.48 mg protein), and 100 μl of 50% trichloroacetic acid. Radioactivity of the 20 000 × *g* supernatant was determined as previously [3].

N-Acetyl-L-tyrosine ethyl ester (ATEE) hydrolysis was measured either potentiometrically with a

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

Recording Titration System 622 (Radiometer) according to [16] or as in [7] adapted for our assay system; in particular, semicarbazide concentration was decreased to 5.3 mM to avoid SMP aggregation, and the incubation mixture was supplemented with potassium cyanide to prevent oxidation of NADH generated.

Hydrolysis of N_α-benzoyl-D,L-arginine-4-nitroanilide (BAPA), N_α-benzoyl-D,L-arginine-1-naphthylamide (BANA), and cytochrome *c* was detected directly in polyacrylamide gels after electrophoretic separation of SMP proteins as proposed in [17,18]. Gels were scanned in a Gilford-250A spectrophotometer.

Protein was determined as in [19] with bovine serum albumin as standard.

3. Results and discussion

For detection of mitochondrial proteolytic activities, ultrasonic SMP were treated with 0.1% Triton X-100 and subjected to electrophoresis in 7% polyacrylamide rod gels with 0.1% Triton X-100; 10–200 μg protein were applied to the gel.

The poorly solubilized membrane material that did not or barely entered the gel was disregarded in all assays. Staining with Coomassie brilliant blue R-250 after 2.5 h electrophoresis revealed 2 major protein zones: one occupied ~1/3 rd of the gel from the start (cathode zone); the other was adjacent to the mobile ion front (anode zone). When the gel was incubated with BANA and post-stained with Fast Garnet GBC, 2 absorption bands were traced densitometrically in the cathode zone (fig.1a). Increase of the separation time to 4 h resulted in a certain migration of the double band towards the anode and appearance of a sharp peak in the middle of the gel (fig.1b). Identical results were obtained with BAPA as substrate. No absorption bands developed if the SMP were preheated for 5 min at 100°C.

This means that either SMP contains 3 different enzymes hydrolyzing BANA and BAPA, or the same hydrolase is under the experimental conditions associated with different membrane components.

In other experiments, proteins of SMP solubilized with Triton X-100 were electrophoresed for 2.5 h, the gels were impregnated with cytochrome *c*, incubated at 35°C and fixed with trichloroacetic acid, as in [18]. In such conditions, hydrolysis of cytochrome *c* manifested itself as the appearance of a translucent

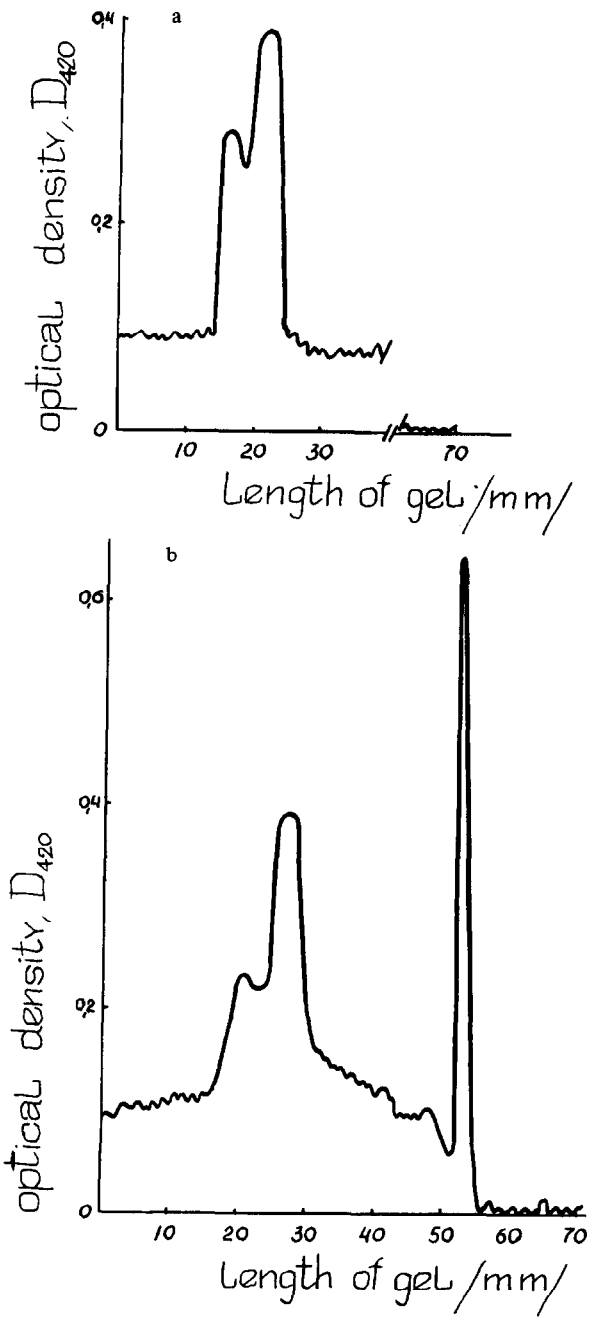

Fig.1. BANA- and BAPA-hydrolyzing activities in submitochondrial preparations from *Saccharomyces cerevisiae*. Inner membrane proteins solubilized with 0.1% Triton X-100 were electrophoresed at 3 mA/tube for 2.5 h (a) or 4 h (b). After the run gels were incubated for 30 min at 35°C with 0.5 mM BANA or BAPA in 20 mM Tris–HCl (pH 8.0). Products of hydrolysis were stained with 0.15% Fast Garnet GBC in 0.33 M acetate buffer (pH 4.5), 5% Triton X-100, for 15 min at room temperature. Stained gels were scanned at 410 nm in a Gilford-250A spectrophotometer.

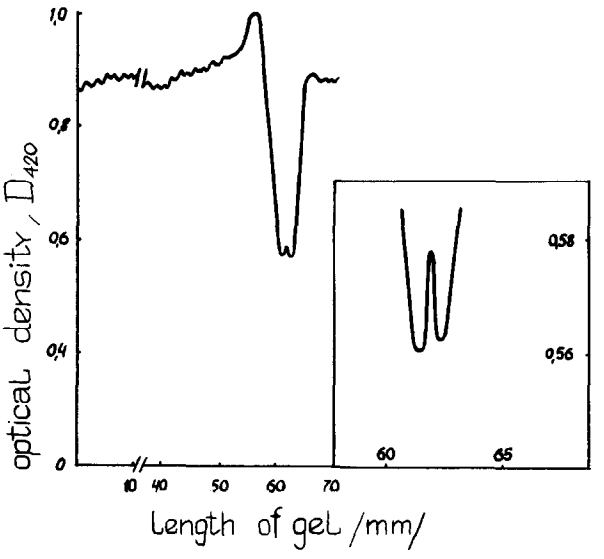

Fig.2. Cytochrome *c* hydrolase activity in yeast submitochondrial preparations. Electrophoretic separation was done as in fig.1 for 2.5 h. Gels were impregnated for 1 h with cytochrome *c* denatured with 8 M urea [18]; then gels were removed from the cytochrome solution, cured for 1 h at 37°C and fixed with 12.5% trichloroacetic acid. Gels were scanned at 410 nm.

band on an opaque background (fig.2). Gel densitometry reveals 2 poorly separated absorption minima, which suggests the existence of 2 enzymes hydrolyzing cytochrome *c*. Proteolysis did not take place when SMP were preheated for 5 min at 100°C.

Thus, yeast mitochondrial inner membrane contains enzyme(s) capable of hydrolyzing cytochrome *c*, and one or several BAPA- and BANA-hydrolyzing enzymes. However, the natural substrates of these hydrolases are as yet unknown.

We have so far failed to determine reliably the inhibitor sensitivity of the hydrolases directly in gels. To obtain such primary information on the identity of the enzymes detected, we attempted to assay the cytochrome *c*-hydrolytic activity in SMP suspensions using labelled cytochrome *c* (section 2). This substrate was found to be cleaved to acid-soluble products at an appreciable rate. Activity is enhanced 1.5-fold or 5-fold by low concentrations (0.05%) of Triton X-100 or SDS, respectively. Proteolysis is inhibited by phenylmethylsulfonyl fluoride (PMSF), *p*-chloromercuribenzene sulfonate (PCMBS), leupeptin, and antipain (table 1). Yeast SMP also hydrolyzes ATEE, a substrate frequently used in assays of chymotrypsin-like enzymes [7,17], at 20–30 nmol/min . mg protein at 38°C. This process displays inhibitor sensitivity similar to that of cytochrome *c* hydrolysis. The same inhibitors had been shown to hinder the breakdown of mitochondrial translation products in yeast cells [5], isolated mitochondria, and ultrasonic SMP [4], as exemplified in table 1. A characteristic feature common for all activities mentioned is their sensitivity to leupeptin, which does not affect any yeast vacuolar enzymes known so far [4,20]. This gives grounds for suggesting that cytochrome *c*-hydrolyzing and ATEE-hydrolyzing activities detected here in mitochondrial inner membrane preparations are due to the enzyme(s) involved in the cleavage of mitochondrial translation products.

The possible relation of these enzymes to the mitochondrial processing machinery awaits further study.

Table 1
Influence of proteinase inhibitors on ATEE- and cytochrome *c*-hydrolyzing activities of SMP and on the breakdown of mitochondrial translation products in yeast mitochondria

Inhibitor	Conc.	Inhibition (%)		
		ATEE hydrolase	Cyt *c* hydrolase	Mitochondrial proteolysis[a]
PMSF	1 mM	86–77	38–51	85
PCMBS	1 mM	95	65	75
PMSF + PCMBS		n.d.[b]	90–100	n.d.
Antipain	2 μg/ml	30–50	61	95
Chymostatin	2 μg/ml	15	n.d.	83
Leupeptin	2 μg/ml	47–50	45–55	55
PMSF + Leupeptin		n.d.	63	n.d.

[a] Quoted from [4]; [b] n.d., not determined

Now it can only be said that the cytochrome *c* hydrolase of SMP is partly (by 20–25%) suppressed by 1 mM *o*-phenanthroline, which has been regarded [11] as an inhibitor of post-translational proteolytic processing. Again, the ATEE hydrolase in SMP is sensitive to aurintricarboxylic acid (30–50% inhibition with 0.5 mM) which has been supposed [2] to block the co-translational processing of the precursor to subunit II of cytochrome *c* oxidase. Nothing can at present be said about the functions of enzymes displaying BAPA- and BANA-hydrolyzing activities.

These results point to the existence of several proteinases in the inner membrane of yeast mitochondria, which appears to be in accord with the current concept of the multiple functions that proteolytic enzymes perform in mitochondrial biogenesis. The exact number of proteinases is yet unknown. The approach proposed here may yield more detailed information along this line if a broader set of substrates are adapted for proteolytic assays in the gel, and conditions are optimized for solubilization and electrophoretic separation of inner membrane proteins.

Acknowledgement

The authors are indebted to Dr A. V. Galkin for stimulating discussions and rendering this paper into English.

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