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Yeast mitochondrial ATP-dependent protease: purification and comparison with the homologous rat enzyme and the bacterial ATP-dependent protease La

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Homogenous ATP-dependent protease has been isolated for the first time from mitochondria of the yeast *Saccharomyces cerevisiae*. The enzyme molecule consists of six 120 kDa subunits. It is a serine protease with an absolute ATP requirement for its activity. Basic enzymatic characteristics of the yeast protease are similar to those of the corresponding rat mitochondrial enzyme and of the *E. coli* protease La. The yeast enzyme immunochemically cross-reacts with the bacterial protease La.

Yeast mitochondria; ATP-dependent protease; Intramitochondrial proteolysis; Protease La; *Saccharomyces cerevisiae*

1. INTRODUCTION

The existence of an ATP-requiring protein for degradation in mitochondria has been known for a long time (reviewed in [1]). The enzyme responsible for the process is the organellar ATP-dependent protease [2,3]. This protease most probably represents a pivotal component of the mitochondrial protein degradation system [2,3]. Isolation and characterization of the enzyme is thus essential for biochemical dissection of the mitochondrial proteolytic machinery. It is also a prerequisite for elucidating the likely role of organellar protein degradation in the overall regulation of mitochondrial biogenesis [4].

The mitochondrial ATP-dependent protease has been purified from bovine adrenal cortex [5] and recently also from rat liver (Kužela and Goldberg, in preparation). The mammalian enzymes resemble in many respects the bacterial ATP-dependent protease La, the product of the *lon* gene in *E. coli* (reviewed in [6]). Surprisingly, no information exists on the mitochondrial ATP-dependent protease of yeast, the most frequently used model in studies on biogenesis of mitochondria. In this paper the procedure for isolation of a homogenous mitochondrial ATP-dependent protease from *Saccharomyces cerevisiae* is described and some of the basic properties of the enzyme are compared with those of the homologous rat enzyme and of the bacterial protease La.

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Abbreviations: AMPPCP, β,γ-methylene adenosine 5-triphosphate; EDTA, ethylenediaminetetraacetic acid; KP_i, potassium phosphate; NEM, *N*-ethylmaleimide; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TLCK, *Nα-p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

2. MATERIALS AND METHODS

2.1. *Cultivation of cells and isolation of mitochondria*

To limit possible problems arising from a contamination of isolated mitochondria by the very active yeast vacuolar proteases, the *Saccharomyces cerevisiae* mutant *pep 949* deficient in two of the major vacuolar proteases [7] was employed. Mitochondria were isolated [8] from spheroplasts [9] of cells grown to an early stationary phase in YEP medium [10] with 2% glucose. The mitochondria were assayed for respiratory control [11], suspended to about 40 mg protein per ml of the preparation medium supplemented with 20% (v/v) glycerol, frozen in liquid nitrogen and stored at −70°C for up to two months.

2.2. *Isolation of the ATP-dependent protease*

The yeast mitochondria (about 2 g protein) were thawed on ice and diluted to about 20 mg protein/ml by buffer A (20 mM Tris-HCl, 0.1 mM EDTA, 1 mM ditiothreitol, 10% (v/v) glycerol, pH 7.9). After adding TPCK and TLCK to 1 mM and aprotinin to 0.1 mg/ml, the matrix fraction was released by 15 min treatment with 0.16 mg Lubrol WX100 per 1 mg protein and recovered as clear supernantant after 30 min centrifugation at 150,000 × *g*. The matrix fraction (about 0.5 g protein) was passed through a 50 ml column of Q Sepharose Fast Flow (Pharmacia) equilibrated with buffer A. The flow through fraction (about 150 mg protein) was made 10 mM with KP_i and applied to a 20 ml column of hydroxylapatite (Bio-Rad) equilibrated with buffer A containing 10 mM KP_i. After eluting the column with a 10 mM to 0.5 M linear gradient of KP_i in buffer A the ATP-dependent protease activity was recovered between 0.2 and 0.3 M KP_i. The active fractions (about 30 mg protein) were dialyzed against buffer A and applied to the FPLC Mono Q HR10/10 column (Pharmacia) equilibrated with the same buffer. On developing the column with a suitable (see Fig. 1) NaCl gradient in buffer A the ATP-dependent protease activity was eluted by about 0.4 M NaCl. The active fractions (about 0.5 mg protein) were concentrated by Centricon (Amicon) and applied to the

FPLC Superose 6 column (Pharmacia) in buffer A with 0.15 M NaCl. The purified ATP-dependent protease (3–5 μg protein) was eluted from the column by the same buffer in a retention volume corresponding to a globular protein with molecular mass of 700 kDa. All the purification steps were done at 0–4°C.

2.3. *Miscellanous*

Protease activity was assayed by determining [^{14}C]-caseine hydrolysis in a mixture containg 50 mM Tris-HCl, pH 7.9 and 10 mM $MgSO_4$ with or without 2 mM ATP as detailed in [4]. Protein estimation [12], SDS-PAGE in 10% acrylamide gels [13] and Western blotting [14] were done according to published procedures. Protein content in the samples of the purified protease was estimated from Coomassie staining of the gels after SDS-PAGE using bovine serum albumin as a standard. The isolated *E. coli* protease La was kindly provided by Prof. A.L. Goldberg and the rat liver mitochondrial ATP-dependent protease was purified from the matrix fraction by a recently developed two-step procedure (Kužela and Goldberg, in preparation). Both the anti-protease La and the anti-rat liver mitochondrial ATP-dependent protease rabbit antisera were obtained in the laboratory of Prof. A.L. Goldberg and in our institute. The immune complexes in Western blots were decorated by the alkaline phosphatase reaction (ProtoBlot, Promega).

3. RESULTS AND DISCUSSION

3.1. *Isolation of the yeast mitochondrial ATP-dependent protease*

The yeast mitochondrial ATP-dependent protease could not be purified by either of the procedures developed for the isolation of homologous bovine [5] or rat (Kužela and Goldberg, in preparation) organellar protease. Therefore, an entirely different protocol for obtaining the homogenous yeast enzyme had to be worked out.

The ATP-dependent protease activity of the yeast mitochondria was detected only in the organellar matrix. The activity was virtually undetectable in the fraction prepared from poorly coupled mitochondria. Therefore, only organelles with a respiratory control index above 2 (NADH as a substrate) were used for isolation of the enzyme.

The relative simplicity of the purification procedure (see section 2, Fig. 1 and Table I) is enabled mainly by the fact that under the conditions used the enzyme does not bind to the Q Sepharose Fast Flow, whereas it is relatively tightly bound to an essentially identical exchanger, the FPLC Mono Q.

The enzyme purified by the described procedure showed a single protein band with a corresponding molecular mass of 120 kDa in SDS-PAGE (Fig. 2A). We do not know at present whether the low yield of the enzyme (about 2 μg from 1 g of mitochondrial protein) reflects a low organellar content of the protease or if it is due to losses during the enzyme purification.

3.2. *Some of the basic properties of the yeast mitochondrial ATP-dependent protease: comparison with the homologous rat enzyme and the bacterial ATP-dependent protease La*

The molecular mass of the enzyme estimated by gel filtration on the FPLC Superose 6 column is about 700 kDa. This finding along with the results of the SDS-PAGE of the isolated enzyme (Fig. 2A) allows for the assumption that the yeast mitochondrial ATP-dependent protease consists of six 120 kDa subunits. This structural feature of the yeast enzyme is similar to that of its mammalian homologs; both the bovine [5] and the rat (Kužela and Golberg, in preparation) mitochondrial ATP-dependent proteases are composed of six 105 kDa subunits. (The smaller of the two prominent peptides seen in the preparation of the rat protease represents a degradation product of the authentic 105 kDa subunit of the enzyme (Kužela and Goldberg, in preparation).) Interestingly, the related bacterial ATP-dependent protease La is a tetramer consisting of 87 kDa subunits [15].

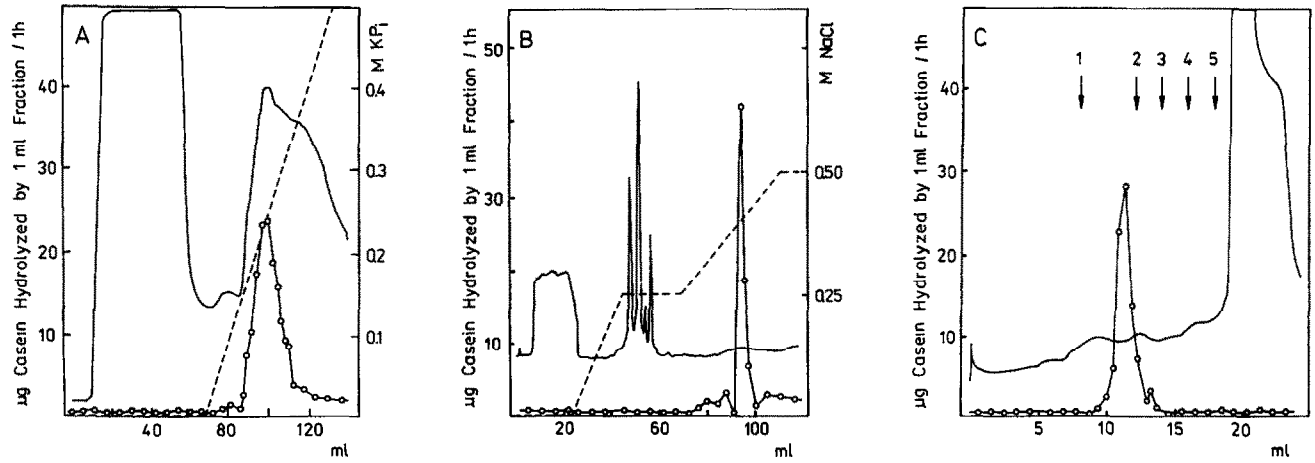

Fig. 1. Protein and protease activity elution profiles during purification of the yeast ATP-dependent protease. Separations on hydroxylapatite (A), FPLC MonoQ (B) and FPLC Superose 6 (C) were done as described in section 2. A_{280} in appropriate scales (—), salt concentration (- - -), protease activity in the presence of 2 mM ATP (- O -). The fractions analyzed did not exhibit any detectable ATP-independent protease activity. The numbered arrows in the part C indicate the positions of molecular mass markers in a similar separation: 1, Blue Dextran (2,000 kDa); 2, thyreoglobulin (669 kDa); 3, ferritin (440 kDa); 4, serum albumin (68 kDa); 5, cytochrome *c* (12 kDa).

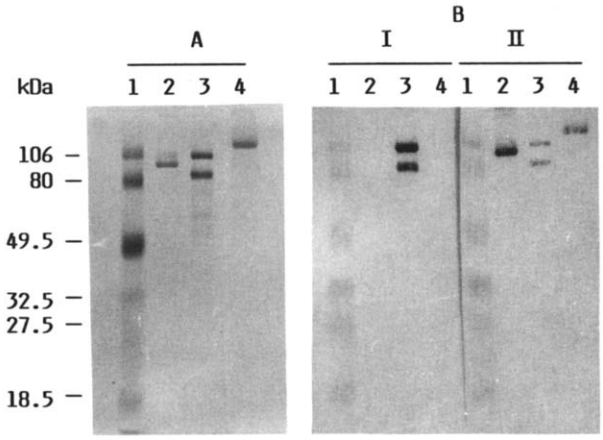

Fig. 2. Electrophoreogram and Western blots of three purified ATP-dependent proteases. Prestained molecular weight standards (Bio-Rad, lanes 1), the *E. coli* protease La (lanes 2), the rat liver (lanes 3) and the yeast (lanes 4) mitochondrial ATP-dependent protease were separated by SDS-PAGE and stained with silver (panel A) or electroblotted to nitrocellulose (panel B). The blots were processed with affinity-purified antibodies against the rat liver mitochondrial ATP-dependent protease (part I) or against the protease La (part II). Approximately equal amounts of the proteases were separated in panel A; in the respective parts of panel B the amount of the protease against which the antibodies were raised was about five times lower than the amounts of the remaining two enzymes.

It cannot be decided at present whether all of the six subunits of the yeast protease are identical as shown for the homologous rat enzyme (Kužela and Goldberg, in preparation) and the bacterial protease La [15].

Among the three ATP-dependent proteases examined in this paper, the yeast enzyme comprises the subunits of the largest size. Considering that the vast majority of the yeast mitochondrial proteins are smaller than their mammalian homologs, the larger size of the yeast protease as compared to its mammalian counterpart is rather exceptional.

Similar to the homologous rat enzyme and the protease La, the yeast mitochondrial ATP-dependent protease has an absolute requirement for ATP and Mg^{2+} (Table II). The enzyme does not exhibit any proteolytic activity in the presence of ADP or AMPPCP, a non-hydrolyzable ATP analog. This indicates that ATP hydrolysis is required for protein degradation by the enzyme. Accordingly, the activity of the yeast protease is sensitive to vanadate (Table II), a potent inhibitor of many ATPases [16]. The pH optimum of the yeast protease is 7.9 and its K_m for ATP is about 40 μM. The K_m values of the mammalian enzyme and of the protease La (10 to 50 μM) determined under identical conditions are in good agreement with published data [4,17]. The inhibition of the yeast enzyme activity by PMSF (Table II) indicates that, similar to the remaining two ATP- dependent proteases tested, the yeast enzyme is also a serine protease. Its high sensitivity to NEM (Table II) points to an essential role of sulfhydryl group(s) in the enzyme activity.

3.3. *Immunochemical relatedness of the yeast mitochondrial ATP-dependent protease to the corresponding rat enzyme and to the bacterial protease La*

Neither the yeast mitochondrial ATP-dependent protease nor the protease La were detected in Western blots by two different polyclonal antibodies against the rat mitochondrial ATP-dependent protease (Fig. 2B, part I). Yet, this cannot be interpreted as a lack of immunochemical cross-reactivity of the three enzymes because both mitochondrial proteases were recognized in the blots by two different anti-protease La antibodies (Fig. 2B, part II). This result shows that the three proteases are related not only functionally but also immunochemically.

Further work on the characterization of the yeast mitochondrial ATP-dependent protease is in progress.

Table I

Summary of the purification of the ATP-dependent protease from yeast mitochondria

Purification step	Protein (mg)	μg [^{14}C]casein hydrolyzed by 1 mg protein per 1 h	
		−ATP	+ATP
Lubrol-treated mitochondria	1,885.0	0.25	0.73
Mitochondrial matrix	528.7	0.39	1.66
Q Sepharose Fast Flow	168.6	< 0.01	8.79
Hydroxylapatite	34.0	< 0.01	16.40
FPLC Mono Q	0.5	< 0.01	188.25
FPLC Superose 6	0.005	< 0.01	2,135.20

Experimental details are given in section 2. Results of a typical purification are presented. The protease activity in the Lubrol-treated mitochondria was determined in the presence of oligomycin (0.5 μg/mg protein).

Table II

Effect of some inhibitors on the activity of the yeast mitochondrial ATP-dependent protease, the rat liver mitochondrial ATP-dependent protease, and the *E. coli* protease La

Inhibitor (concentration)	2 mM ATP	Activity (%) of the protease from		
		Yeast	Rat liver	*E. coli*
PMSF (5 mM)	+	47.7	51.2	54.7
NEM (1 mM)	+	< 1.0	22.0	53.1
EDTA (25 mM)	+	< 1.0	< 1.0	< 1.0
Vanadate (5 mM)	+	40.2	38.5	63.3
None	−	< 1.0	< 1.0	< 1.0

Experimental details are given in section 2. Results of a typical experiment are presented.

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