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NEW CHELATOR-SENSITIVE PROTEASES IN MATRIX OF YEAST MITOCHONDRIA

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Summary: Proteases in yeast mitochondria were studied using fluorogenic synthetic substrates containing methylcoumaryl amide (MCA). Among the eleven substrates which are commonly employed for several types of proteases, Leu-MCA, Arg-MCA, Boc-Gln-Arg-Arg-MCA and Boc-Phe-Ser-Arg-MCA were found to be highly susceptible to proteases in mitochondria. All these proteases were localized in the matrix and sensitive to o-phenanthroline but not to phenylmethylsulfonyl fluoride or iodoacetate. The analysis of hydrolyzed products of Boc-Gln-Arg-Arg-MCA indicated that the peptide was cleaved at the site between Gln and Arg. These results demonstrate that there exist chelator-sensitive aminopeptidase(s) and endopeptidases in the matrix of yeast mitochondria.

The protein constituents of mitochondria turn over at heterogeneous rates and intramitochondrial proteolysis is suggested to be responsible for the protein turnover at least in the mitochondrial internal compartments such as inner membrane and matrix (see review, ref.1). There have been two lines of evidence demonstrating the presence of intramitochondrial proteolysis systems. One of them is a matrix-localized soluble protease that processes the cytoplasmically-synthesized precursors of mitochondrial proteins to their mature forms (2,3). The other is an ATP-dependent proteolysis which seems to act on proteins with abnormal conformation (4). These two proteolytic systems are likely to be involved in the specific functions within the mitochondria. It can be assumed, therefore, that mitochondria should have other proteases or proteolysis systems for the turnover of proteins constituting the organelle. There are several reports on other proteases in the mitochondria of yeast and mammalian cells as reviewed in ref.1. However, little precaution has been paid to the possible contamination of the mitochondrial fraction with other organelles. As the first step toward investigating the turnover of

Abbreviations used in this paper: MCA, 4-methylcoumaryl-7-amide; Boc, t-butoxycarbonyl; Suc, succinyl; Cbz, carbobenzoxyl; AMC, 7-amino-4-methylcoumarine; PMSF, phenylmethylsulfonyl fluoride; HPLC, high-performance liquid chromatography.

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mitochondrial proteins, we started to survey proteases in the mitochondria with precaution against contamination by extra-mitochondrial proteases. The present paper reports on the proteases in yeast mitochondria detected using fluorogenic 4-methylcoumaryl-7-amide (MCA) -containing substrates. Most of the proteases are localized in the matrix and are sensitive to a metal chelator, o-phenanthroline. One of them has an interesting substrate specificity of cleaving the amino side of paired basic amino acid residues in the substrate.

Materials and Methods: The wild type Saccharomyces cerevisiae strain D273-10B (ATCC,25657) was used throughout the study. Methods for growing the cells and converting them to spheroplasts were similar to those described previously (5). Spheroplasts were washed three times in 1.2 M sorbitol, 10 mM Tris-HCl,pH 7.5, resuspended in 0.6 M sorbitol, 10 mM Tris-HCl,pH 7.5, and then broken with a Dounce-type homogenizer as described previously (6). The homogenate was centrifuged successively twice at 1,500xg for 5 min and once at 12,000xg for 10 min. The resulting pellet containing mitochondria (mitochondrial fraction) was washed by two cycles of centrifugation at 1,500xg for 5 min and at 12,000xg for 10 min. The supernatant resulting from the first centrifugation at 12,000xg was served as post-mitochondrial supernatant. Sucrose density gradient centrifugation was performed as described by Douglas et al.(7). Mitochondria isolated by differential centrifugation were subfractionated by a controlled osmotic shock method of Daum et al.(6) except that the procedure to shrink the mitochondria before sonic oscillation was omitted.

Protease activity was measured in a 0.5 ml reaction mixture containing 20 μM of aminoacyl-MCA or peptidyl-MCA in 50 mM Tris-HCl,pH 7.5, and an enzyme source of 3-50 μg as protein. When membrane fractions of mitochondria or other organelles were used as an enzyme source, they were solubilized in 0.5% of Triton X-100 prior to the assay. Aminopeptidase M (18 mU/ml) was included in the reaction mixture, when endoproteolytic activities were measured using peptidyl-MCA substrates whose amino termini are blocked with the t-butoxy-carbonyl (Boc), succinyl (Suc) or carbobenzoxyl (Cbz) group. After the incubation at 30 °C for 30-70 min, the amount of released 7-amino-4-methyl-coumarine (AMC), a proteolytic product of aminoacyl-MCA or peptidyl-MCA was measured fluorophotometrically with excitation and emission at 380 and 460 nm, respectively. To test whether the cleavage occured at the peptide bond between the amino acids or that between the amino acid and MCA in the peptidyl-MCAs, aminopeptidase M was not included in the reaction mixture but 50 μM of bestatin was added to inhibit the endogenous aminopeptidase activity. One unit of activity was defined as the amount required to release one nmole of AMC per min under the assay conditions used. To test the inhibition of proteolysis by o-phenanthroline which also inhibits the added aminopeptidase M, the following procedures were employed. After the incubation for proteolysis in the presence of 1 mM o-phenanthroline, the whole assay mixture was heated to 100°C for 10 min and cooled. After the action of o-phenanthroline was stopped by addition of 1.2 mM $CoCl_2$, aminopeptidase M was added and the incubation was continued at 30°C for 30 min. High-performance liquid chromatography (HPLC) was carried out with a cation exchange column, Mono S HR5/5.

Published methods were employed for measuring protein (8) and assaying enzymes, carboxypeptidase Y (9), NADPH cytochrome c reductase (10), cytochrome c oxidase (11), kynurenine hydroxylase (12), cytochrome b_2 (13) and fumarase (14). Synthetic substrates of amino acyl-MCA and peptidyl-MCA were purchased

from Bachem AG, Switzerland and Peptide Institute Inc.,Osaka, Japan, aminopeptidease M from Sigma, and Mono S column from Pharmacia.

Results and Discussion

In order to detect proteases in yeast mitochondria, it is essential to prepare mitochondria free from other organelles, especially vacuoles which have a large amount of various proteases. Figure 1 shows the results on purity of mitochondria and some proteolytic activities residing in the organelle. When tested by sucrose density gradient centrifugation followed by marker enzyme assay, the mitochondrial fraction isolated by differential centrifugation contained practically no microsomes (NADPH cytochrome c reductase) or vacuoles (carboxypeptidase Y). These two organelles were predominantly recovered in the post-mitochondrial supernatant. A small amount of NADPH cytochrome c reductase cofractionated with mitochondria (cytochrome c oxidase) might be due to the enzyme inherent in mitochondria (15). The sedimentation profiles of activities hydrolyzing Leu-MCA, Boc-Gln-Arg-Arg-MCA and Boc-Phe-Ser-Arg-MCA were very similar to that of mitochondrial marker enzyme but not to those of other organelle marker enzymes, indicating that the protease hydrolyzing these MCA-substrates are located in the mitochondria.

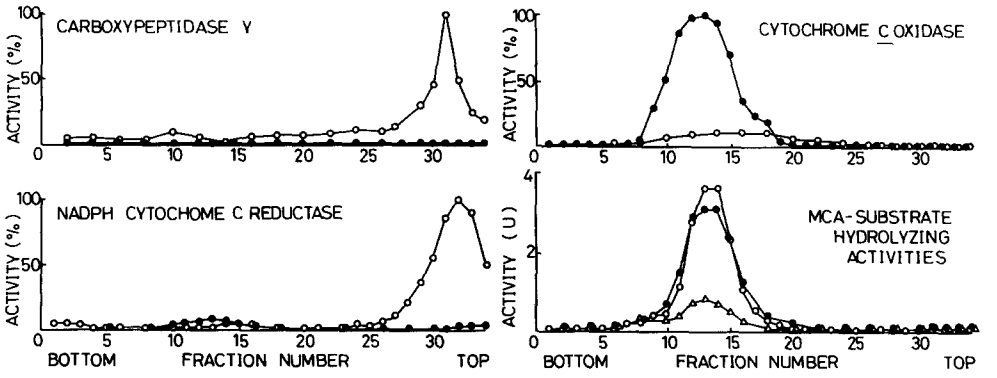

Fig. 1. Mitochondrial fraction isolated from yeast cells is free of microsomes and vacuoles, and contains proteolytic activities hydrolyzing MCA-substrates: Mitochondrial fraction(9 mg protein) and post-mitochondrial supernatant(12 mg protein) were each layered on a linear 20-70% sucrose gradient and centrifuged at 20,000 rpm at 4°C for 15 hr in a Kontron TST 23.38 rotor. Samples of 1 ml each were collected and assayed. Marker enzymes assayed were carboxypeptidase Y (vacuoles), NADPH cytochrome c reductase (microsomes),and cytochrome c oxidase (mitochondria). The ordinate denotes the activity of marker enzyme as expressed in terms of the percentage in each fraction of the total activity in both mitochondrial and post-mitochondrial supernatant fractions. ●-●, mitochondrial fraction; o-o, post-mitochondrial supernatant. Fractions obtained by sucrose density gradient centrifugation of mitochondrial fraction were assayed as described in Materials and Methods for activities hydrolyzing Leu-MCA (o-o), Boc-Gln-Arg-Arg-MCA (●-●) and Boc-Phe-Ser-Arg-MCA (△-△).

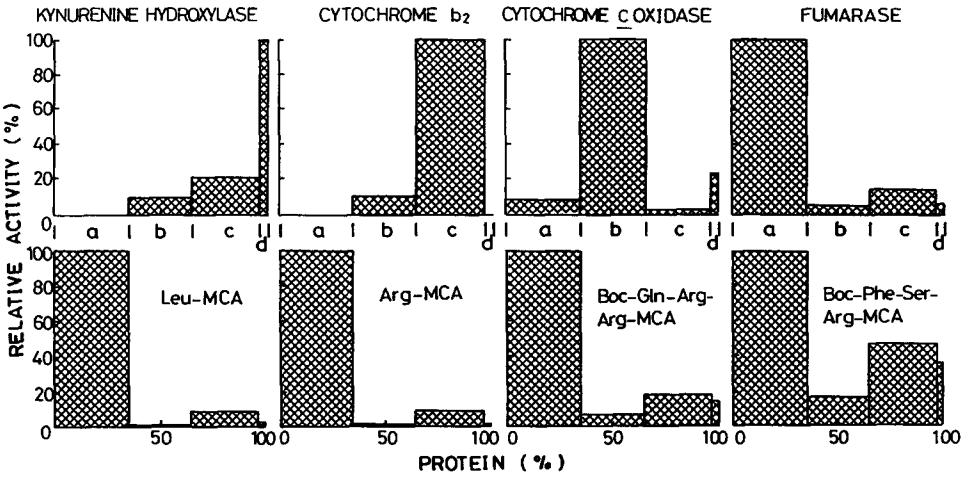

Fig. 2. Distribution of marker enzymes and protease activities in subfractions of yeast mitochondria: Mitochondria were subfractionated as described in Materials and Methods and assayed for marker enzymes of mitochondrial subfractions (upper panels) and for activities hydrolyzing MCA-substrates (lower panels). Marker enzymes assayed were fumarase (matrix), cytochrome c oxidase (inner membrane), cytochrome b_2 (intermembrane space), and kynurenine hydroxylase (outer membrane). The ordinate denotes the specific activity of each enzyme as expressed in terms of percentage of the highest specific activity in the subfractions. The actual values for 100% of MCA-substrate hydrolyzing activities are as follows, Leu-MCA, 1.96 U/mg; Arg-MCA,1.82 U/mg; Boc-Gln-Arg-Arg-MCA, 3.28 U/mg; Boc-Phe-Ser-Arg-MCA, 1.38 U/mg. The abcissa denotes the percentage of mitochondrial proteins recovered. a, matrix; b, inner membrane; c, intermembrane space; d, outer membrane.

To identify the intramitochondrial localization of the proteases, mitochondria isolated by differential centrifugation were subfractionated into four compartments; outer membrane, intermembrane space, inner membrane and matrix (Fig.2). The marker enzyme assay indicated a good separation of the compartments, though a small amount of outer membrane contaminated in the intermembrane space and inner membrane fractions. The spectrum of protease activity hydrolyzing Leu-MCA, Arg-MCA, or Boc-Gln-Arg-Arg-MCA was very similar to that of fumarase, indicating that these proteolytic activities are localized in the mitochondrial matrix. The activity hydrolysing Boc-Phe-Ser-Arg-MCA was also found mainly in matrix. These results further exclude the possibility that the proteases detected are originally in the cytosol or other organelles and adsorbed to the mitochondria during the cell homogenization, since such contaminants should be mainly recovered in the mitochondrial outer compartments but not in the matrix that are enclosed by inner membrane.

Table I shows protease activities in the mitochondrial subfractions on peptidyl-MCA substrates other than those described above. Boc-Glu-Lys-Lys-MCA, Suc-Leu-Leu-Val-Tyr-MCA and Cbz-Arg-Arg-MCA were preferentially hydrolyzed by matrix fraction. Both matrix and intermembrane space fractions

Table I. Protease Activities in Mitochondrial Subfractions

Substrate	Specific Activity (U/mg x10)			
	OM	IMS	IM	MX
Boc-Glu-Lys-Lys-MCA	1.77	1.38	0.68	3.58
Suc-Leu-Leu-Val-Tyr-MCA	0.09	0.29	0.04	1.56
Cbz-Arg-Arg-MCA	0.83	0.29	0.33	1.48
Boc-Val-Pro-Arg-MCA	3.39	4.40	1.49	5.25
Suc-Ala-Ala-Pro-Phe-MCA	0.58	1.01	0.30	0.95
Suc-Ala-Pro-Ala-MCA	0.09	0.19	0.04	0.18
Pro-Phe-Arg-MCA	0	0	0	0

Experimental conditions were the same as for Fig.1 except that MCA-substrates listed were tested. OM, outer membrane; IMS, intermembrane space; IM, inner membrane; MX, matrix.

contained activities that cleave Boc-Val-Pro-Arg-MCA, Suc-Ala-Ala-Pro-Phe-MCA and Suc-Ala-Pro-Ala-MCA. Pro-Phe-Arg-MCA was not hydrolyzed by any of the mitochondrial subfractions.

To characterize the mitochondrial proteases, effects of protease inhibitors were examined using the four substrates which were highly susceptible to the proteases in mitochondrial matrix. As shown in Table II, the hydrolysis of all the four substrates was inhibited by o-phenanthroline but not by iodoacetate or phenylmethylsulfonyl fluoride (PMSF). Bestatin, a specific inhibitor of aminopepetidases, inhibited the hydrolysis of Leu-MCA and Arg-MCA; this indicates that mitochondrial matrix contains a chelator-sensitive aminopeptidase(s) as well as endopeptidases and therefore has the capacity to digest proteins to the level of amino acids. When incubated with matirix fraction in the presence of bestatin, Boc-Phe-Ser-Arg-MCA and Boc-Gln-Arg-Arg-MCA released practically no fluorescent AMC, indicating that they are

Table II. Effects of inhibitors on proteolytic activities in the mitochondrial matrix

Substrate	Remaining Activity (%)			
	Iodoacetate[a]	PMSF[a]	o-Phenanthroline[b]	Bestatin[a]
Leu-MCA	102	98	8	3.4
Arg-MCA	98	100	7	13
Boc-Gln-Arg-Arg-MCA	91	97	9	0.3
Boc-Phe-Ser-Arg-MCA	94	88	31	0

a. To a reaction mixture containing mitochondrial matrix, iodoacetate, phenylmethylsulfonyl fluoride (PMSF) or bestatin was added to a final concentration of 1 mM, 1 mM or 50 μM, respectively. After 10 min preincubation at room temperature, the reaction was started by adding the MCA-substrate.
b. Detailed assay conditions are described in Materials and Methods.

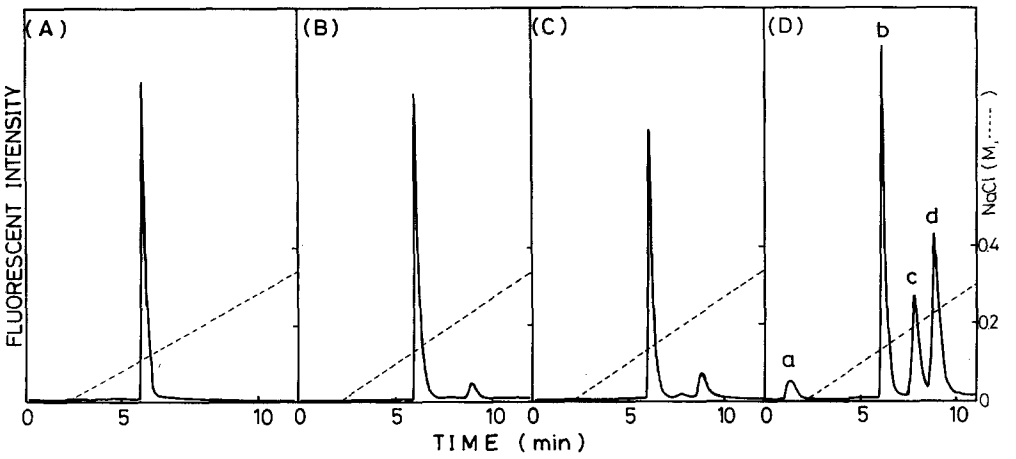

Fig. 3. Analysis of proteolysis products of Boc-Gln-Arg-Arg-MCA: The incubation for proteolysis of Boc-Gln-Arg-Arg-MCA was performed as described in Materials and Methods except that the enzyme source was a mitochondrial soluble fraction (8 μg as protein) which was prepared by sonic oscillation of mitochondria followed by centrifugation at 200,000xg for 60 min to remove membrane fractions and that the concentration of bestatin in the reaction mixture was 10 μM. At 0 min (A), 30 min (B) and 60 min (C) of incubation, 10 μl aliquots were taken and mixed with 40 μl of 10 mM HCl to stop the reaction. The mixture was diluted 5-fold with 60% ethanol, 20 mM Tris-HCl,pH7.5 (column equilibration buffer) and 10 μl of the mixture was applied to a Mono S HR5/5 column. The elution was carried out by linearly increasing the concentration of NaCl in the above buffer. The eluate was monitored fluorophotometrically with excitation and emission at 325 nm and 395 nm, respectively. Panel D represents the elution profile of mixture containing an equal amount of AMC (a), Boc-Gln-Arg-Arg-MCA (b), Arg-MCA (c) and Arg-Arg-MCA (d).

cleaved at the site between the amino acids other than that between Arg and MCA in these substrates. Each of these two proteolytic activities seems to be catalyzed by a distinct enzyme since two activities were separated from each other by DEAE-cellulose column chromatography (not shown). When effect of bestatin on the release of AMC was similarly tested with other substrates listed in Table I except Pro-Phe-Arg-MCA, none of them except Suc-Ala-Pro-Ala-MCA yielded AMC.

To determine at which site Boc-Gln-Arg-Arg-MCA was cleaved by the protease in mitochondrial matrix, the proteolytic products of this substrate were analyzed by HPLC. As shown in Fig.3, a fluorescent peak was eluted from the column and was identified as Arg-Arg-MCA with an authentic standard. When the proteolysis incubation was carried out for a longer period, a small amount of Arg-MCA appeared in the eluate (Fig.3,C). Proteolysis by mitochondrial matrix of other substrates, Cbz-Ala-Arg-Arg-MCA and Boc-Gln-Lys-Lys-MCA, also yielded Arg-Arg-MCA and Lys-Lys-MCA, respectively (not shown). These results demonstrate that the matrix of yeast mitochondria contains a chelator-sensitive metal endopeptidase that cleaves the amino side of paired basic residues such as Arg-Arg or Lys-Lys in the synthetic substrates. This metal protease seems different from the previously reported ATP-dependent protease

and precursor-processing protease. The ATP-dependent protease is a PMSF-sensitive serine protease (4). The precursor-processing protease is a 115-kilodalton protein presumably composed of two 55-kilodalton subunits (2) whereas, according to our preliminary studies, the protease hydrolyzing Boc-Gln-Arg-Arg-MCA is composed of a single 96-kilodalton peptide.

Precursor peptides of most hormones and neurotransmitters contain paired basic residues and the proteolytic scission at these residues releases the physiologically active peptides. Attention has recently been paid to proteases specific for these paired basic residues. Two kinds of such proteases have been detected in yeast cells. Mizuno and Matsuo purified a soluble serine protease which cleaves various peptide substrates at a peptide bond between the paired basic residues (16). Julius et al.(17) and Achstetter and Wolf (18) reported a membrane-bound, presumably microsomal, metal protease which hydrolyzes the carboxyl side of paired basic residues of synthetic substrates such as Boc-Gln-Arg-Arg-MCA or Cbz-Tyr-Lys-Arg-4-nitroanilide. Both proteases were supposed to process a prepro-alpha-mating factor. The proteolytic activity of mitochondrial matrix shown in Fig.3 appears to be catalyzed by an enzyme different from these proteases since it hydrolyzes the peptide bond at the amino side of paired basic residues of peptides.

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