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A NOVEL PROTEINASE ASSOCIATED WITH MITOCHONDRIAL MEMBRANES

James F. Hare

Department of Biochemistry
School of Medicine
University of Oregon Health Sciences Center
Portland, Oregon 97201

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SUMMARY

Mitochondrial inner membranes catalyzed the endoproteolytic release of radioactively labelled polypeptide fragments of ^{125}I labelled insulin covalently attached to agarose beads. Demonstration of this proteolytic activity was dependent upon detergent and inhibited by a variety of proteinase and sulfhydryl group inhibitors. Unlike three previously described peripheral mitochondrial proteinases, the insulin hydrolyzing mitochondrial proteinase was resistent to extraction with high salt but solubilized with a non-ionic detergent, indicating its integral association with the membrane.

Proteinases are involved in cell processes as diverse as the processing of viral polypeptide precursors (1), cleavage of newly translated polypeptides in preparation for their export from the cell (2) or insertion into membranes (3), and regulation of enzyme levels by turnover (4). Little is known, however, of the function, cellular location, and regulation of cell proteinases. The mitochondrion is a particularly attractive setting in which to examine proteinase function since this organelle exhibits protein synthesis and degradation independent of that occurring in the cytoplasm and is isolated from lysosomal and other cellular influences by a limiting membrane system.

Isolated mitochondria display auto-proteolysis as measured by the continual release of amino acids into their suspension medium (5). Despite evidence to the contrary (6), indirect evidence suggests that mitochondria possess a proteolytic system (7,8,9,10). Indeed, three reports have recently identified proteinases from the mitochondrial fractions of mammalian cells (11,12,13). In this report, I describe an additional proteinase which is detected by solid phase assay and is distinct from previously isolated enzymes. The unique properties possessed by this enzyme suggests an unusual role for it in the

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metabolism of mitochondrial proteins.

MATERIALS AND METHODS

Mitochondria were prepared from overnight fasted Sprague-Dawley male rats (300 g) by a high yield method (14). Low speed centrifugations were combined and comprised the nuclear fraction while the combined post-mitochondrial supernatents were centrifuged for 1 hr at 100,000xg to separate the microsome from the soluble fraction. Mitoplasts, intermembrane fraction, and outer membrane were prepared as described (15) but mitoplasts were sonicated at 20 mg of protein per ml for four x 15 sec intervals at about 100 watts with a one-half inch disruptor horn and centrifuged at 100,000xg for 1 hr to separate matrix and inner membrane fractions.

^{125}I-insulin coupled Sepharose was synthesized essentially as described (16). 20 mg of bovine insulin (Sigma) was dissolved in 2.0 ml of 0.2M sodium carbonate, pH 9.0 (carbonate buffer). The following were added in sequence: 1 mCi carrier free Na^{125}I (ICN) in 40 µl, 0.05 mg lactoperoxidase (Boehringer), and 10 µl 0.088M H_2O_2. The reaction was allowed to proceed for 2 hr at room temperature and the protein dialyzed for 20 hr at 4° against three 125 ml changes of carbonate buffer. The iodinated protein was then dialyzed for an additional 3 hr against 125 ml of 0.2M citrate, 0.7M NaCl (pH 6.0) at 4° during which time the protein solution became turbid. The labelled insulin (34 µCi/mg protein) was added to 1 g of washed Sepharose 4B-CNBR (Pharmacia) and incubated with end over end rotation at 23° for 2 hr. Glycine was added to 1M and end over end rotation continued for 5 hr at 4°. The insulin labelled agarose beads were then washed on a glass sintered filter at room temperature unless otherwise indicated with 100 ml volumes of the following: carbonate buffer, 0.1M NaCl; carbonate buffer, 0.1M NaCl, 8 M urea; 1% sodium lauryl sulfate (90°); carbonate buffer, 0.1M NaCl, 1% Triton X-100; carbonate buffer, 0.1M NaCl; 0.1M Tris-HCl (pH 8.0). The washed substrate beads (6 mg protein/ml Sepharose) were resuspended in 20 ml 0.1M Tris-HCl (pH 8.0), 2.5 mM NaN_3 and stored at 4° until used (within three weeks).

The proteinase assay reaction mixture was contained in 1.5 ml Eppendorf centrifuge tubes and had the following composition: 0.1M Tris-HCl (pH 8.0), 0.25% Triton X-100, and routinely 20-200 µg of enzyme protein in a total volume of 0.9ml. One or more blanks in which enzyme was excluded was run with each series of assays. An aliquot of substrate beads equal in volume to 0.1 times the total final assay volume was removed from cold storage, boiled in 1% sodium lauryl sulfate for 2 min, and washed consecutively with 200 volumes of water, 1% Triton X-100, water, 0.1M Tris-HCl (pH 8.0), and finally resuspended to a total volume of 0.1 times the total assay volume with 0.1M Tris-HCl (pH 8.0). Assays were started by the addition of 0.1 ml of washed, diluted substrate beads (3.3 µCi ^{125}I and 0.1 mg protein) and the tubes rotated end over end at 60 rpm and 23°. for 15 min. The reaction was stopped by rapid centrifugation of the suspension on a microfuge (Brinkmann) and three 0.1 ml samples were taken and their radioactivity determined by scintillation counting in 90% Triton-toluene (17). Counting efficiency was 54%. One unit of proteinase activity corresponds to 10^3 CPM released per min at 23°.

Marker enzymes were assayed by the following methods: monoamine oxidase (18), cytochrome c oxidase (19), acid phosphatase (20), malate dehydrogenase (21), and glucose-6-phosphatase (22). The method of Jusic et al. (12) measured N-acetyl-tyrosine ethyl esterase. Protein was estimated by the method of Lowry et al. (23) using crystalline bovine serum albumin as a standard.

RESULTS

Proteinase assay - Proteinase function has been traditionally assessed by colorimetric assays which measure esterase activity or the release of trichloroacetic acid soluble amino acid residues. These methods, however, are not sensitive enough to unearth previously unidentified cellular proteinases and do not distinguish between exo- and endoproteolysis. Solid phase assays in which commercially available proteins are radioactively labelled to high specific activity with ^{125}I and covalently coupled to solid support matrices have been described (24,25). Radioactivity released after incubation with enzyme and removal of insoluble substrate is a measure of peptide bond cleavages. These assay systems offer the advantages of sensitivity and detection of endoproteolysis. Ideally, the protein substrate should be attached to the solid support at only one or a few residues to minimize the chances of non-productive cleavages. Bovine insulin coupled to cyanogen bromide activated agarose beads at pH 6.0 should serve as an ideal proteinase substrate since at this pH, insulin is covalently coupled to the agarose support by only its B-chain alpha-amino group (16), thereby exposing two trypsin and several chymotrypsin sensitive sites to a soluble proteinase. The ready iodination of insulin followed by covalent attachment to the support should provide a proteolytic substrate of defined sequence and high sensitivity.

^{125}I insulin-Sepharose was synthesized and tested as a substrate for trypsin and rat liver mitochondria. Trypsin released radioactive polypeptides from the beads, but mitochondria were only active after Triton X-100 was added to the incubation buffer. Radioactivity released per minute for both trypsin and mitochondria was linear with concentration of enzyme (Fig. 1A) and time (data not shown) and could detect 5 ng of trypsin in the standard 15 min incubation period. At higher enzyme concentrations or longer incubation times, the radioactivity released deviates from linearity (Fig. 1B). That addition of more substrate beads overcomes this problem suggests that substrate is limiting. In these studies enzyme concentrations were kept low enough to measure on the

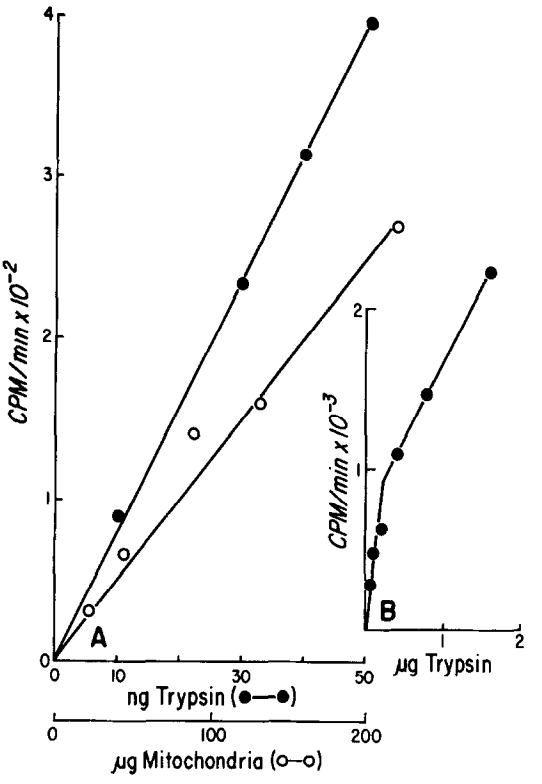

Fig. 1. Hydrolysis of ^{125}I-insulin-Sepharose as a function of trypsin and mitochondria concentration. Incubation was for 15 min at 23°. Trypsin incubation medium included 1.5mM $MgCl_2$.

linear portion of the curve in Fig. 1A (100-400 cpm released per min above a blank rate of 75-100 cpm released per min).

pH optimum studies of mitochondrial solid phase activity showed an optimum between pH 8.0 and 8.5. Neither monovalent cations (Na^+ or K^+ at 0.1M) nor divalent cations (Mg^{+2}, Ca^{+2}, or Mn^{+2} at 1mM) were stimulatory or inhibitory. 1.0 mM Zn^{+2} inhibited both trypsin and mitochondrial activity, indicating an effect on substrate rather than proteinase. Other detergents (cholate, deoxycholate, Tween 80, and Lubrol WX) could substitute for Triton X-100 with varying and generally lower degrees of effectiveness. Proteolytic release was increasingly stimulated by Triton concentrations up to 0.20%.

Proteolytic digests from trypsin and the mitochondria fraction appeared heterogeneous in size when chromatographed on a Sephadex G-75 column in the

presence of 0.25% sodium lauryl sulfate. Released, radioactive polypeptide fragments eluted between the void and included volume and were clearly longer than single amino acids, demonstrating that the enzyme is acting endoproteolytically. Thus, insulin presents several substrate sites with possibly different susceptibility.

Cellular and mitochondrial localization of proteinase activity - A rat liver homogenate was fractionated by preparative centrifugation to obtain nuclear, microsomal, mitochondrial, and soluble fractions. In addition, since lysosomes sediment with the mitochondrial fraction, mitochondria were treated with 1.2% digitonin to remove contaminating smooth and outer mitochondrial membranes, thereby obtaining the mitoplast fraction (15). Marker enzymes and proteinase were determined in each fraction and the results shown in Fig. 2. Purification of proteinase activity into the mitochondrial fraction parallels that of cytochrome c oxidase and the lysosomal marker, acid phosphatase, although some proteinase activity remains in the high speed, microsomal pellet. Digitonin treatment concentrates the proteinase into the mitoplast fraction along with cytochrome oxidase, while it quantitatively solubilizes the lysosomal marker, acid phosphatase.

Fractionation of mitochondria into four compartments as described by Greenawalt (15) was followed with marker enzyme activities and the results presented in Fig. 3. Distribution of proteinase followed a pattern identical to that of cytochrome c oxidase, both activities being purified with the inner membrane fraction.

Inhibitor specificity - The mitochondrial proteinase was sensitive to sulfhydryl group reagents added directly to the reaction mixture or added to membranes before their centrifugation at 100,000xg for 60 min, resuspension, and assay (Table I). EDTA stimulated the proteinase by 150-220%. Chymotrypsin inhibitor L-1-tosylamide-2-phenylethylchloromethyl ketone as well as the trypsin inhibitor p-aminobenzamidine inhibited the enzyme; but the serine proteinase inhibitor, phenylmethanesulfonylfluoride, was without effect on activity (Table I).

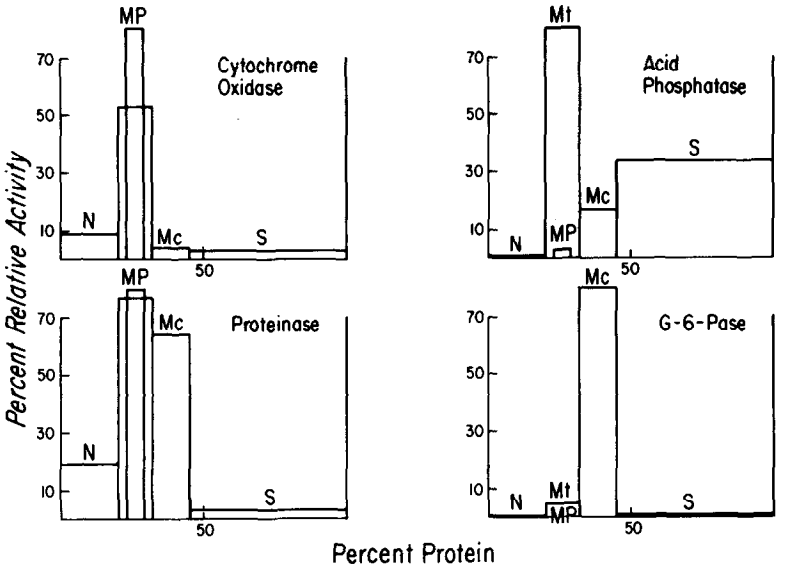

Fig. 2. Cellular distribution of insulin hydrolyzing proteinase. Fractionations were as described in Methods. Specific activities of marker enzymes were set at 80% and were as follows: cytochrome oxidase, 0.48 µmoles cytochrome c/min/mg mitoplast protein at infinite cytochrome c concentration; acid phosphatase, 0.13 µmoles phosphate/min/mg mitochondrial protein; glucose-6-phosphatase, 0.30 µmoles phosphate/min/mg microsomal protein; proteinase, 1.29 units/min/mg mitoplast protein. Fractions are abbreviated from left to right: N, nuclear fraction; Mt, mitochondria fraction; Mp, mitoplast fraction of mitochondria; Mc, microsomes; S, soluble fraction.

Solubilization from the membrane - Recent reports (11,12,13) describe the solubilization of proteinases from mitochondrial membrane preparations with 0.5M phosphate at pH 8.5. The proteinase described in this report was investigated to see if it too could be solubilized by phosphate or whether it might exhibit a different association with the membrane. Inner mitochondrial membrane preparations were extracted by 0.5M potassium phosphate or 1M KCl, by itself, in addition to Triton X-100, or in sequence with Triton X-100 (Table 2). Confirming the observations of Jusic et al. (12) N-acetyl-tyrosine ethyl esterase was in majority extracted after a 4° two-day incubation in 0.5M phosphate. This same treatment, however, resulted in extraction of only 10% of the insulin hydrolyzing proteinase while the rest remained in the residue. 1M KCl was also unsuccessful in extracting the insulin hydrolyzing proteinase but in combination with Triton

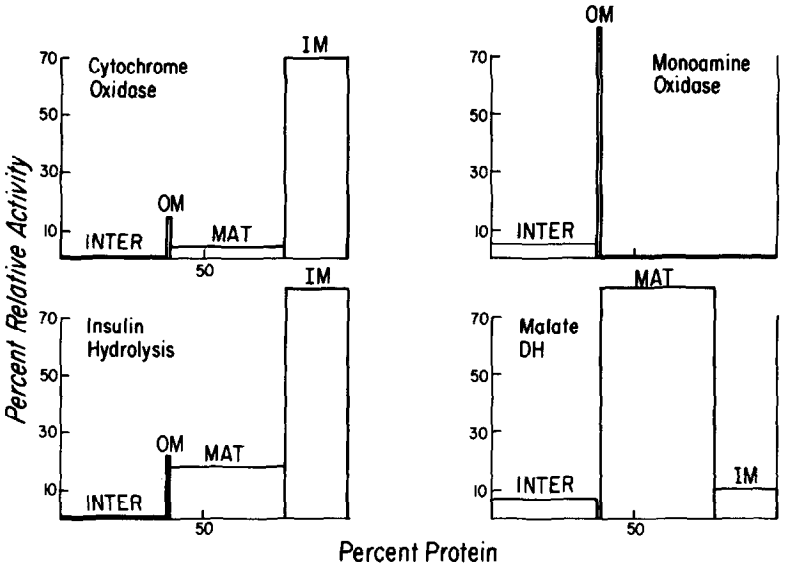

Fig. 3. Mitochondrial distribution of proteinase activity. Fractionation was according to (15) as modified in Methods. Specific activity of marker enzymes were set at 80% and were as follows: cytochrome c oxidase, 6.17 µmoles cytochrome c/min/mg inner membrane protein; monoamine oxidase, 212 nmoles benzylamine/min/mg outer membrane protein; malate dehydrogenase, 16.0 µmoles NADH/min/mg matrix protein; and proteinase (insulin hydrolase), 3.23 units/min/mg inner membrane protein. Fractions are abbreviated from left to right: inter, intermembrane fraction; OM, outer membrane; mat, matrix fraction; IM, inner membrane fraction.

solubilized 71% of the enzyme. Extraction of the membranes sequentially with 1M KCl and 2% Triton resulted in a three-fold enrichment of the proteinase in the Triton extract.

DISCUSSION

The membrane bound endoproteinase identified in this report is clearly localized in the mitochondrial inner membrane on the basis of fractionation studies, thereby showing its non-identity to proteinases associated with lysosomes (26), microsomes (25,27), and plasma membranes (28,29). Its integral membrane localization and detergent stimulation rules out identity to soluble proteinases. The insulin specific proteinase also differs from previously described mitochondrial proteinases (11,12,13) in its possessing the following characteristics: (1) resistence to salt extraction from mitochondrial membrane preparations; (2) detergent stimulation of proteolytic activity; and (3) inhi-

TABLE I

EFFECT OF VARIOUS REAGENTS ON PROTEINASE ACTIVITY

Treatment	Concentration	Percent Control Activity: Mitochondrial Proteinase	Percent Control Activity: Trypsin
--	--	100	100
Minus Triton X-100	--	6	100
EDTA	1.0mM	212	80
Sodium lauryl sulfate	0.25%	0	--
o-Phenanthroline	1.0mM	98	--
Phenylmethanesulfonylfluoride	1.0mM	84	22
Soybean trypsin inhibitor	50µg/ml	96	0
p-Aminobenzamidine	1.0mM	6	0
	0.1mM	57	12
L-1-tosylamide-2-phenylethyl-chloromethyl ketone	0.1mM	4	115
	0.01mM	35	--
N-ethylmaleimide	10µM	1	102

TABLE II

EXTRACTION OF PROTEINASES FROM INNER MEMBRANE

Extraction Conditions	Recovered Units: ^{125}I-Insulin[1]	Recovered Units: ATEE[2]	Protein (mg)
0.5M PO_4 (pH 8.5)			
Extract	6.0	144	4.2
Residue	59.0	49	16.0
1M KCl (1 volume)			
Extract	9.7	-	6.3
Residue	45.0	-	9.3
1M KCl + 1% TX100			
Extract	41.0	-	13.9
Residue	17.0	-	5.9
1M KCl (5 volumes) - 2% TX100			
Extract (KCl)	11.0	-	11.0
Extract (TX100)	29.0	-	4.3
Residue	17.0	-	3.1

[1] One unit equals 10^3 CPM released per minute.

[2] N-acetyltyrosine ethyl ester hydrolysis; one unit equals 1 nmole NADH per minute.

bition by sulfhydryl group reagents but not by phenylmethanesulfonylfluoride.

Since mitochondria demonstrate many functions that might require proteolysis, it is not surprising that they contain proteinases. Such proteinases may function to (1) process newly translated polypeptides synthesized in the mitochondria or cytoplasm and directed towards or across the mitochondrial membrane; (2) remove excess mitochondrially translated polypeptides not incorporated correctly into the membrane; or (3) selectively degrade mitochondrial proteins to control their steady state levels. A detergent requirement for the mitochondrial membrane proteinase suggests that this enzyme prefers a hydrophobic environment for expression of activity and *in vivo* may utilize membrane proteins as substrates.

Comparison of specific proteolytic activity expressed by purified trypsin and the mitochondrial membrane proteinase suggests that the latter comprised 0.1% or more of mitochondrial protein. Efforts are under way in this laboratory to purify the enzyme from Triton extracts of 1M KCl washed inner mitochondrial membranes.

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