J. Biochem. **123**, 408-415 (1998)

Evidence for a Novel ATP-Dependent Protease from the Rat Liver Mitochondrial Intermembrane Space: Purification and Characterisation

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Received for publication, September 12, 1997

An ATP-dependent protease in the intermembrane space of rat liver mitochondria, MISP I (mitochondrial intermembrane space protease), was partially purified and characterised. The protease complex has a molecular mass of 200 kDa and appears to be an oligomeric enzyme complex. The proteolytic activity of the enzyme can be stimulated up to 3-fold by Mg^{2+}ATP. The K_m for ATP is 200 μM. Nucleoside triphosphates, but not ADP, AMP, or nonhydrolysable ATP analogues, can substitute for ATP. The protease exhibits multicatalytic properties with chymotrypsin-like, peptidyl-glutamyl-hydrolysing, and trypsin-like activities. Of the latter the trypsin-like activity is not enhanced by ATP. In addition to the hydrolysis of fluorogenic peptide substrates the protease is able to degrade radiolabeled model proteins. The ATP-dependent mitochondrial protease was characterised as a cysteine protease sensitive to hemine. The cross reactivity of an anti-human-S4 antibody raised against an ATPase subunit of the PA700 complex with a component of MISP I indicated a structural relationship. Furthermore, ATP-agarose-binding assays revealed the connection of the peptide hydrolysing activity with an ATP binding domain. The data presented here and a comparison with known ATP-dependent mitochondrial proteases demonstrated that MISP I represents a novel ATP-dependent protease in the mitochondrial intermembrane space of rat liver.

Key words: ATP, cysteine protease, mitochondrial intermembrane space, proteolysis, rat.

Proteolysis is an essential tool for regulating cellular functions at the post-translational level. Mammalian cells contain two major distinct proteolytic pathways—lysosomal and non-lysosomal proteolytic systems. The cathepsins, the proteases of lysosomes, are responsible for the breakdown of proteins that enter a cell from the extracellular milieu or some intracellular proteins (*1*). Numerous observations have indicated that the major part of the protein turnover in cells is catalysed through non-lysosomal mechanisms. Different proteins have widely differing half-lives, ranging from a few minutes to many days. The controlled degradation of proteins inside cells seems to be one important part of the maintenance of protein homeostasis. Therefore, the selective recognition of proteins that are destined for degradation by proteases is an important part of the controlled protein turnover in mammalian cells. One of the first known and today the best investigated pathway for the selective recognition and degradation of proteins is the ATP- and ubiquitin-dependent cytosolic protein degradation by the 26S protease. This pathway first reported by Hershko and coworker (for a review see Ref. *2*) has been detected in many different tissues. The central enzyme of this pathway is the 26S protease complex consisting of a catalytic core, the 20S protease or proteasome, and two regulatory complexes, the 19S regulator (*3*), also called PA700 (*4*). Mammalian cells have developed a system for the recognition and labeling with ubiquitin of proteins that are to be degraded. This ubiquitin-conjugating system consists of at least three different types of enzymes responsible for the selective poly-ubiquitinylation of proteins (*5*). The poly-ubiquitinylated proteins are recognized by the 26S protease and degraded by the enzyme complex. For a number of specific regulatory proteins, such as cyclins A and B (*6*), tumor suppressor proteins such as p53 (*7*), proto-oncoproteins such as c-Mos (*8*), and transcription factors such as NF-κB/IκB (*9*), and also for abnormal proteins (*2*), degradation *via* the ubiquitin- and ATP-dependent proteolytic pathway has been described.

The existence of various proteolytic systems in compartments besides the cytosol and lysosomes is generally accepted today. A number of papers have described proteolytic systems in the nucleus (*10*), the endoplasmic reticulum (*11*), the plasma membrane (*12*), and the mitochondria.

Previously, several proteases from different mitochon-

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Abbreviations: Cyt *c*, cytochrome *c*; E-64, *N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucine-4-guanidino-butylamide; MCA, 7-amino-4-methylcoumarin; MISP I, ATP-dependent mitochondrial intermembrane space protease; βNA, β-naphthylamide; NEM, *N*-ethylmaleimide; Suc-LLVY, succinyl-Leu-Leu-Val-Tyr; z-LLE, benzyloxycarbonyl-Leu-Leu-Glu; z-PFR, benzyloxycarbonyl-Pro-Phe-Arg.

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drial compartments were described, several of them being ATP-dependent. For mitochondria from mammalian cells, Desaultes and Goldberg (*13, 14*) and Watabe and Kimura (*15, 16*) reported the identification and characterisation of an ATP-dependent mitochondrial matrix protease, later identified as a homolog of the Lon protease from *Escherichia coli* (*17, 18*). Additionally, the involvement of at least one ATP-dependent metalloprotease in the degradation of incomplete translation products of mitochondrial protein synthesis localized in the inner mitochondrial membrane was demonstrated (*19-21*). Langer and coworkers (*21-23*) reported that the proteases of the mitochondrial inner membrane are members of a known ATPase family, the AAA-family, containing a highly conserved ATPase module (*24, 25*). In the mitochondrial inner membrane, a 850 kDa complex consisting of at least two proteolytic enzymes called Yta10p and Yta12p is formed (*23*). YTA10, YTA11, and YTA12 encode, besides the ATPase module, HEXXH, a conserved metal-binding motif, indicating the metalloprotease activity of the translated protein (*21-23*). The catalytic sites of the Yta10p and Yta12p proteases are located on the matrix side of the inner membrane, whereas Yta11p exhibits a proteolytic action in the intermembrane space (*22*).

The presence of a novel ATP-dependent proteolytic activity in the mitochondrial intermembrane space in rat liver was reported previously by us (*26*). Here we describe the partial purification and enzymatic characterisation of this ATP-dependent protease from rat liver mitochondria. The results and a comparison with known ATP-dependent mitochondrial proteases demonstrated the existence of MISP I, a novel ATP-dependent protease, in the mitochondrial intermembrane space.

MATERIALS AND METHODS

Isolation and Fractionation of Mitochondria—For one preparation, the liver mitochondria isolated from six fed male Wistar rats (250-300 g body weight) were subfractionated into the matrix, inner membrane fraction, outer membrane fraction, and intermembrane space as described previously (*26*). Marker enzyme activities for these compartments were measured by standard methods (*26*). Protein concentrations were determined according to Bradford (*27*).

Isolation and Purification of the ATP-Dependent Protease—The intermembrane space fraction, which exhibited the highest specific ATP-stimulable proteolytic activity, was applied to a DEAE-cellulose (Whatman DE52) column (2.5 × 16 cm) equilibrated with 10 mM Tris-HCl (pH 7.5), 0.1 mM dithiothreitol, 0.1 mM EDTA, and 20% glycerol. Proteins were eluted with a linear gradient of 0-0.4 M KCl at the flow rate of 0.5 ml/min, then 4.5-ml fractions were collected. Fifty microliters of every third fraction was used for proteolytic assaying with Suc-LLVY-MCA as the substrate.

The ATP-dependent proteolytic activity was eluted in two peaks, which were separately pooled and concentrated to a final volume of 2 ml by ultrafiltration. Pool I (fractions 40-61) and pool II (fractions 62-77) were each diluted to 5% glycerol and then layered onto a 58-ml glycerol gradient [10-40%, v/v, in 10 mM Tris-HCl (pH 7.5), 0.1 mM dithiothreitol, and 0.1 mM EDTA]. After centrifugation at 50,000 × g in a SW 25.2 rotor for 30 h at 4°C, the gradients were collected in 2-ml fractions from the bottom of the tubes. Twenty microliters of every second fraction was used for proteolytic assaying with Suc-LLVY-MCA as the peptide substrate. The peak I and peak II activities from the DEAE-column sedimented at the same positions in the glycerol gradient. Fractions 25-27 of the glycerol gradients, containing the highest proteolytic activity, were pooled and separated with an FPLC-system on a MonoQ column (HR5/5-Pharmacia) equilibrated with 10 mM Tris-HCl (pH 7.0), 0.1 mM dithiothreitol, and 0.1 mM EDTA. The adsorbed proteins were eluted with a linear gradient of 0-0.4 M KCl, 1-ml fractions being collected. Twenty microliters of each fraction was used for the protease assay. Fraction 16 was reapplied to the MonoQ column and then the proteins were eluted under the same conditions, except for the use of a slower gradient.

Fraction 22 obtained on re-chromatography on MonoQ, with the highest proteolytic activity, was used for further characterisation of the ATP-dependent proteolytic activity.

Assaying of Peptidase Activity ("Protease Assay")—For assaying, the fluorogenic substrates (Suc-LLVY-MCA, z-PFR-MCA, and z-LLE-βNA) were incubated with 20-50 μl of a sample for 30 min at 37°C. The standard reaction mixture contained the following components in a total volume of 0.3 ml: 10 mM Tris-HCl (pH 7.0), 0.5 mM dithiothreitol, 200 μM Suc-LLVY-MCA as the peptide substrate in the presence or absence of 5 mM ATP, and 5 mM $MgCl_2$. The reaction was stopped by adding of 300 μl of ice-cold absolute ethanol and 3 ml 125 mM borate buffer (pH 9.0). The fluorescence of the reaction products was monitored with 380 nm excitation and 440 nm emission for MCA, or 335 nm excitation and 410 nm emission for βNA.

To determine the pH-dependence of the ATP-dependent proteolytic activity the following buffer systems, with a final concentration of 10 mM, were used: phthalate-NaOH (pH 4.0-5.5), imidazole-HCl (pH 6.0-7.0), and Tris-HCl (pH 7.5-9.0).

Polyacrylamide Gel Electrophoresis—Protease samples were analysed by discontinuous PAGE under non-denaturing conditions, and continuous PAGE under denaturing conditions.

The non-denaturing gels consisted of 4% stacking gels that had been polymerised with riboflavin (0.04%) in 50 mM Tris (pH 6.8) and 2.3% (w/v) sucrose, and the 4.5-15% gradient resolving gels were cast in TBE-buffer [90 mM Tris, pH 8.3, 80 mM boric acid, 0.08 mM EDTA, and 2.5% (w/v) sucrose], polymerised with ammonium persulfate (0.04%). Samples were diluted with 5% glycerol, and then electrophoresed for 4 h at 100 V and 4°C in TBE buffer. High molecular weight marker proteins (Pharmacia) were used for non-denaturing gel electrophoresis. SDS-PAGE was performed as described by Laemmli (*28*) in 10% resolving and 4.5% stacking gels. The molecular weight markers used were as follows: lysozyme, 14 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 46 kDa; bovine serum albumin, 69 kDa; phosphorylase *b*, 97.4 kDa; and myosin, 200 kDa. After electrophoresis and/or substrate overlaying (see below), the gels were stained by the silver stain method of Blum *et al.* (*29*).

Substrate Overlaying—Protease activity was detected in non-denaturing gels by overlaying the gels with 10 mM Tris (pH 7.0), 0.1 mM dithiothreitol, 5 mM Mg^{2+}ATP, and 200

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μM Suc-LLVY-MCA, followed by incubation of the gels at room temperature for 5-10 min. The gels were washed three times for 3 min each in water. The hydrolysis product—free MCA—was visually detected under UV irradiation.

Immunoblots—Proteins were transferred from SDS-PAGE gels to nitrocellulose membranes using a Bio-Rad Trans-Blot apparatus. The nitrocellulose membranes were blocked with 5% non-fat dry milk in 1.5 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 115 mM NaCl, and 0.4% Tween 20 (phosphate-buffered saline) prior to incubation in milk buffered with phosphate containing the antibody used. The secondary antibody used for detection was POD anti-rabbit IgG. The nitrocellulose blots were developed by means of the ECL-assay (Amersham), and then exposed to Kodak X-OMAT AR films for chemiluminescence detection.

RESULTS

Isolation and Purification of the ATP-Dependent Protease, MISP I—MISP I was purified from the mitochondrial intermembrane space fraction by DEAE-chromatography at pH 7.5. The elution profile of the Suc-LLVY-MCA-hydrolysing activity of a representative preparation in the presence and absence of 5 mM ATP is shown in Fig. 1A. The ATP-dependent proteolytic activity was detected in two not well separated peaks (peak I and peak II), which were eluted between 0.14 and 0.27 M KCl. Although the ratio of peak I and peak II varied between isolations, peak I was generally more abundant than peak II. ATP stimulated the

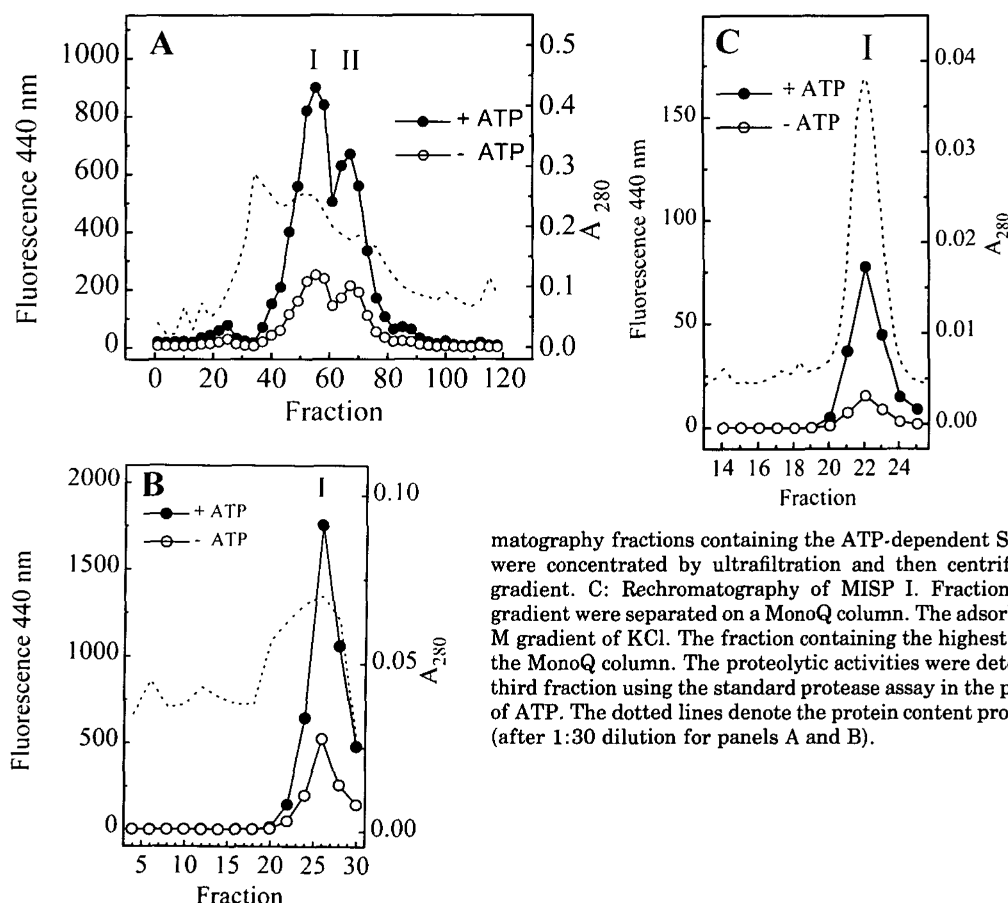

Fig. 1. **Isolation of the ATP-dependent protease of mitochondrial intermembrane space from rat liver, MISP I.** The fractionation of mitochondria and the further purification steps are described in detail under "MATERIALS AND METHODS." A: Fractionation of proteins from the mitochondrial intermembrane space on DEAE-cellulose. The intermembrane space fraction from the mitochondria of six male Wistar rats was chromatographed on a 2.5 × 16-cm column of Whatman DE52. Proteins were eluted with a linear gradient of 0-0.4 M KCl in 4.5-ml fractions. B: Glycerol density gradient sedimentation of MISP I. The DEAE-chromatography fractions containing the ATP-dependent Suc-LLVY-MCA-hydrolysing activity were concentrated by ultrafiltration and then centrifuged in a 10-40% glycerol density gradient. C: Rechromatography of MISP I. Fractions 25-27 from the glycerol density gradient were separated on a MonoQ column. The adsorbed proteins were eluted with a 0-0.4 M gradient of KCl. The fraction containing the highest proteolytic activity was reapplied to the MonoQ column. The proteolytic activities were determined in aliquots of each second or third fraction using the standard protease assay in the presence (+ATP) or absence (−ATP) of ATP. The dotted lines denote the protein content profiles estimated as the A_{280} absorbance (after 1:30 dilution for panels A and B).

TABLE I. **Purification of the ATP-dependent protease, MISP I.** The data shown are for a representative preparation and are related to the fractions used during preparation. One unit of proteolytic activity is defined as 1 nmol of Suc-LLVY-MCA hydrolysed per min at 37°C.

Preparation step	Total protein (mg)	Total units ($nmol \cdot min^{-1}$)		Specific activity ($nmol \cdot mg^{-1} \cdot min^{-1}$)		Recovery (%)		Purification factor		ATP-stimulation factor
		+ATP	−ATP	+ATP	−ATP	+ATP	−ATP	+ATP	−ATP	
Mitochondria	547.20	1,061.57	306.43	1.94	0.56	100.0	100.0	1.0	1.0	3.5
Intermembrane space	24.32	609.70	105.06	25.07	4.32	57.4	34.3	12.9	7.7	5.8
DEAE-chromatography[a]	16.80	399.17	102.80	23.76	6.12	37.6	33.5	12.2	10.9	3.9
Glycerol gradient	1.62	118.58	51.09	73.17	31.54	11.2	16.7	37.7	56.3	2.8
MonoQ	0.39	86.29	35.30	221.25	90.50	8.1	11.5	114.0	161.6	2.4
Rechromatography on MonoQ	0.04	12.55	4.11	313.85	102.81	1.2	1.3	161.8	183.6	3.0

[a] Beginning with this preparation step the data are only related to the further purification of peak I.

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hydrolysis of the fluorogenic peptide substrate about 3-fold. Peak I (fractions 40-61) and peak II (fractions 62-77) obtained on DEAE-chromatography were separately pooled and desalted by ultrafiltration. Further separation was performed by sedimentation on glycerol gradients (Fig. 1B). The peak I and peak II Suc-LLVY-MCA-hydrolysing activities sedimented at the same positions. The materials in the two protein fractions differed in the absolute proteolytic activity and the stimulation by ATP. The ATP-stimulation was 3-fold for peak I and 2-fold for peak II. According to the sedimentation, the Suc-LLVY-MCA-hydrolysing activity is that of a protein with a molecular mass of approximately 200 kDa.

For further purification, column fractions exhibiting proteolytic activity were subjected to MonoQ chromatography. The proteolytic activity present in peak I was eluted between 0.18-0.28 M KCl and was stimulated 4-fold by ATP. Fraction 16 was reapplied to the MonoQ-column, however, a slower increasing salt gradient was used (Fig. 1C). A single major protein peak containing Suc-LLVY-MCA-hydrolysing activity stimulatable up to 3-fold with 5 mM ATP was eluted in fraction 22. Peak II exhibited identical elution behaviour on the MonoQ column. In agreement with the initial proteolytic activity with the glycerol gradient of peak II, lower proteolytic activity of peak II in the presence or absence of 5 mM ATP was recovered after rechromatography on the MonoQ-column in comparison to peak I.

The proteolytic activities recovered at different preparation steps are summarised in Table I. The purification resulted in 160-fold enrichment of the ATP-dependent protease of the mitochondrial intermembrane space.

For further characterisation of the ATP-dependent protease, peak I was analysed with regard to its protein content and proteolytic activity (Fig. 2). Four individual protein bands were detectable after separation by non-denaturing gel electrophoresis (Fig. 2A). Of these, however, only one protein band (band 3) exhibited proteolytic activity in the overlay assay.

The SDS-PAGE analysis of peak I demonstrated the presence of at least 14 different polypeptides (Fig. 2C).

Gel filtration on a Superdex 200-column did not result in further purification, since all four proteins obtained on non-denaturing gel electrophoresis were eluted as one peak (Fig. 3).

Characterisation of the ATP-Dependent Protease, MISP I—Based on the elution profile of the marker proteins on the Superdex 200-column, the molecular mass of MISP I was estimated to be about 200 kDa (Fig. 3).

To determine the type of proteolytic activity, the effects of various inhibitors on the hydrolysis of Suc-LLVY-MCA were analysed (Table II). Thiol-blocking reagents (NEM and leupeptin) and an inhibitor (E64) of cysteine proteases each had a drastic effect on the proteolytic activity suggesting that the ATP-dependent MISP I is a sulfhydrylprotease. The enzyme activity was not affected by PMSF, *o*-phenanthroline or pepstatin. Hemin, a known inhibitor of the cytosolic 26S protease complex, inhibited, at a concentration of 25 μM, the ATP-dependent and -independent

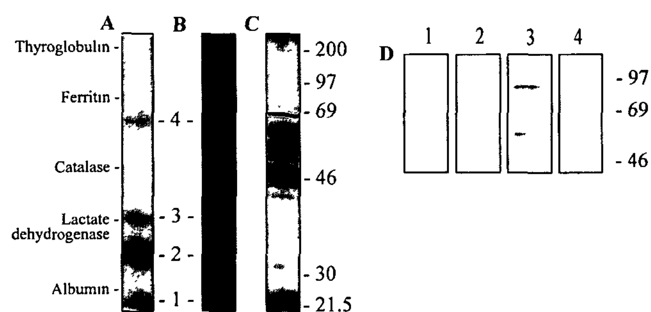

Fig. 2. **Electrophoretic analysis of the purified ATP-dependent protease, MISP I.** Fraction 22 on the rechromatography (see Fig. 1C) was analysed by non-denaturing gel electrophoresis. Gels were either stained with silver (A) or overlaid with Suc-LLVY-MCA (B). In panel C, SDS-PAGE of fraction 22 on the rechromatography is shown. Panel D: The four bands obtained on the non-denaturing gel electrophoresis were separately analysed on an SDS-gel and examined for cross reactivity with an anti-human-S4 antibody (generously supplied by C. Gordon). The markers in panel A indicate the non-denaturing gel electrophoresis of a high molecular weight calibration kit (Pharmacia). The molecular weights indicated in panels C and D were obtained by separation of the SDS-PAGE rainbow-markers (Amersham).

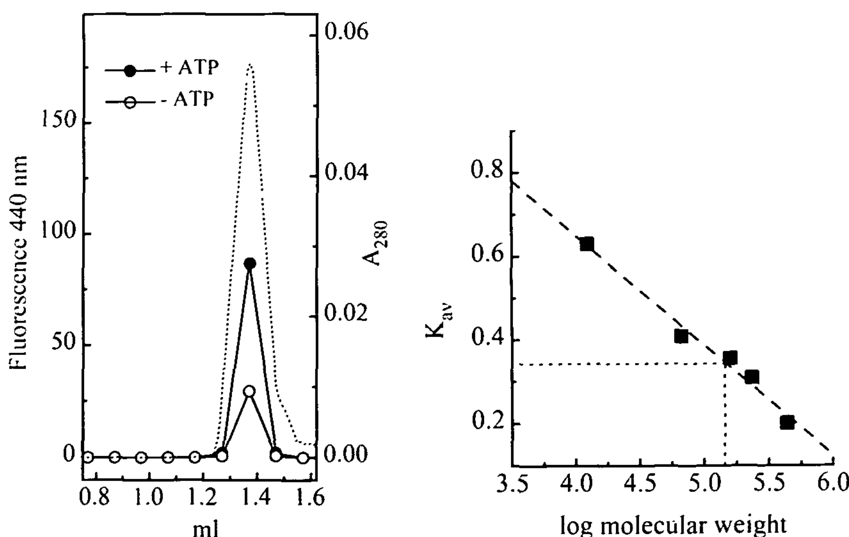

Fig. 3. **Size exclusion chromatography of MISP I.** Fraction 22 on the rechromatography on MonoQ was concentrated with Centricon30 and Microcon30 (Amicon), and then applied to a Superdex200 column (PC 3.2/30; Pharmacia) in a Smart-system (Pharmacia). Proteins were eluted with 10 mM Tris, pH 7.0, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1 M KCl. 5-μl aliquots of the 100-μl fractions collected were used for determination of the proteolytic activity with 200 μM Suc-LLVY-MCA as the substrate. In the left panel the size exclusion chromatography of the concentrated fraction 22 on the rechromatography is demonstrated. The right panel shows the molecular weight determination of the protease using a calibration curve for the Superdex200 column. Molecular weight markers were: ferritin (440 kDa); catalase (232 kDa); aldolase (158 kDa); bovine serum albumin (67 kDa); and cytochrome *c* (12.4 kDa).

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proteolysis almost completely.

The catalytic properties of the ATP-dependent MISP I were examined using various flourogenic peptides (Table III). The enzyme exhibited both chymotrypsin-like (Suc-LLVY-MCA) and peptidylglutamyl-hydrolysing (z-LLE-βNA) activities which can be stimulated by ATP (Table III). Interestingly, the trypsin-like activity, as indicated by the hydrolysis of z-PFR-MCA, was not stimulated by ATP (Table III).

The kinetic properties of the ATP-dependent protease, MISP I, were assayed by titration with different substrate concentrations. A K_m of 116 μM and a V_{max} of 38 nmol$\cdot$mg$^{-1}\cdot$min^{-1} were determined for the chymotrypsin-like activity with Suc-LLVY-MCA as the substrate (Fig. 4A).

To analyse the protein-degrading properties of the protease, radiolabeled model proteins were used. The data shown in Fig. 5A demonstrate that of the three tested proteins only casein was degraded faster in the presence of Mg^{2+} ATP. The pH optimum for the hydrolysis of casein by the protease was pH 8.0 (Fig. 5B).

For further characterisation of ATP-dependent MISP I, the effect of substitution of ATP with other energy-rich nucleoside triphosphates as well as AMP, ADP, and a non-hydrolysable ATP-analog (β,γ-CH_2-ATP) on the energy-dependent hydrolysis of Suc-LLVY-MCA, as shown in Table IV, was examined. The hydrolysis of

TABLE II. **Inhibitors of the ATP-dependent protease MISP I.** The enzyme and the indicated inhibitors were preincubated for 10 min at 37°C in the absence of a fluorogenic peptide. The reactions were initiated by the addition of Suc-LLVY-MCA. The data are the means for 3-5 independent experiments with SD of always less than 5%.

Inhibitor	Concentration	% inhibition	
		+ATP	−ATP
NEM	1 mM	99	99
Leupeptin	10 μM	77	80
	100 μM	94	92
E64	280 μM	77	81
	1.12 mM	95	95
Hemin	25 μM	94	96
Pepstatin	1 μM	0	0
	5 μM	12	12
PMSF	5 mM	1	0
	10 mM	15	34
o-Phenanthroline	1 mM	0	0

TABLE III. **Comparison of the different proteolytic activities of MISP I.** The proteolytic activities were measured as described under "MATERIALS AND METHODS" with the exception of the various peptide substrates which were used at 20 μM. The values represent the mean±SD fluorescence at 380/440 nm for MCA and 335/410 nm for βNA for three independent experiments.

Peptide substrate	Fluorescence		ATP-stimulation factor
	+ATP	−ATP	
Suc-LLVY-MCA	65.3±2.9	20.1±0.6	3.2
z-LLE-βNA	11.2±0.3	5.1±0.1	2.2
z-PFR-MCA	166.9±6.6	167.4±0.8	1.0

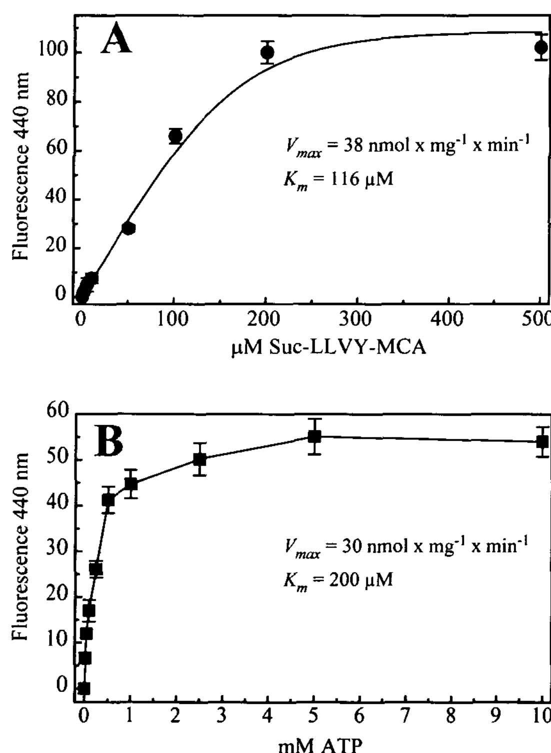

Fig. 4. **Kinetic properties of MISP I.** The kinetic properties of MISP I with different concentrations of the fluorogenic peptide substrate, Suc-LLVY-MCA (panel A), and various ATP-concentrations (panel B). In panel B the indicated fluorescence values demonstrate the difference between the proteolysis in the presence and the absence of ATP. The conditions are given under "MATERIALS AND METHODS." The data represent the means±SD for four independent experiments. K_m and V_{max} were calculated by fitting the data with non-linear regression using the "SIMFIT" software (*47*).

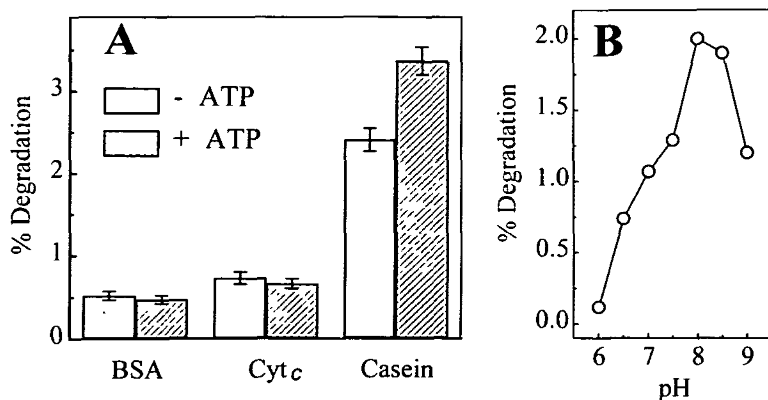

Fig. 5. **Degradation of model proteins by MISP I.** The model proteins, bovine serum albumin (BSA), cytochrome *c* (Cyt *c*), and casein, were radiolabeled by reductive methylation as described by Jentoft and Dearborn (*30*). The degradation was performed under the standard conditions using a substrate concentration of 0.4 μg/ml (panel A). Panel B shows the pH-dependence of the casein degradation. Percent degradation was calculated as described by Pacifici *et al.* (*31*). The values represent the means±SD for five independent experiments in the case of panel A. The mean values for five independent experiments with SD of less than 7% are presented in panel B.

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TABLE IV. **Effects of nucleotides on the hydrolysis of Suc-LLVY-MCA by MISP I.** One hundred percent represents the proteolytic activity in the presence of 5 mM ATP and 5 mM $MgCl_2$ with Suc-LLVY-MCA as the substrate, with the exception that instead of ATP the indicated nucleotides were used. The data are the means for three independent experiments with SD of less than 10%.

Compounds	Proteolysis (%)
None	35
5 mM $MgCl_2$	38
5 mM ATP	36
5 mM ATP+5 mM $MgCl_2$	100
5 mM CTP+5 mM $MgCl_2$	83
5 mM UTP+5 mM $MgCl_2$	50
5 mM GTP+5 mM $MgCl_2$	44
5 mM AMP+5 mM $MgCl_2$	33
5 mM ADP+5 mM $MgCl_2$	33
5 mM β,γ-CH_2-ATP+5 mM $MgCl_2$	29
5 mM ATP+1 mM ADP+6 mM $MgCl_2$	37
5 mM ATP+5 mM ADP+10 mM $MgCl_2$	33

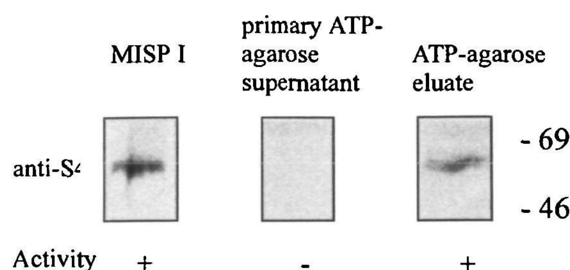

Fig. 6. **Immunoblot of MISP I after ATP-agarose fractionation.** MISP I was incubated with ATP-agarose at 4°C overnight in binding buffer (10 mM Tris, pH 7.0, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 5 mM $MgCl_2$). The ATP-agarose was centrifuged and the supernatant (primary ATP-agarose supernatant) was used for immunoblotting. The ATP-agarose was washed five times with the binding buffer. The bound protein was eluted using the binding buffer supplemented with 5 mM ATP (ATP-agarose eluate). The samples were analysed under the standard electrophoretic and immunoblot conditions (see "MATERIALS AND METHODS"). The antibody used was anti-human-S4 antibody. The figures indicate the molecular weights of protein standards. "Activity" indicates the presence of Suc-LLVY-MCA-hydrolysing activity under the standard conditions, as given under "MATERIALS AND METHODS."

Suc-LLVY-MCA by the protease was maximally stimulated by Mg^{2+}ATP, followed by Mg^{2+}CTP, Mg^{2+}UTP, and Mg^{2+}GTP. The peptide hydrolysis was not stimulated by ADP or AMP. The ATP-analog, β,γ-CH_2-ATP, was not able to stimulate the peptide hydrolysis, suggesting the necessity of ATP-hydrolysis for ATP-stimulation. The addition of ADP to the standard reaction mixture together with ATP inhibited the ATP-dependent proteolysis, indicating substrate competition. Furthermore, hydrolysis of Suc-LLVY-MCA was measured in dependence of the ATP concentration (Fig. 4B). Assuming a Michaelis-Menten-type reaction, ATP-dependent proteolysis, a K_m of 200 μM and a V_{max} of 30 nmol·mg^{-1}·min^{-1} were determined.

To examine the relationship of the novel ATP-dependent MISP I to other ATP-dependent mammalian proteases we used an antibody against the S4-ATPase subunit of the PA700 complex of the 26S protease. This 26S protease subunit possesses an ATP binding site (*32*). Of the four proteins detected on non-denaturing gel electrophoresis, only the protein exhibiting peptidase activity (protein band 3) reacted with the anti-S4 antibody (Fig. 2D), suggesting the presence of a ATP-binding module in the novel ATP-dependent protease from the mitochondrial intermembrane space.

To obtain further information about the ATP-binding properties of MISP I, we performed an ATP-agarose binding assay (Fig. 6). After elution of the adsorbed proteins, the Suc-LLVY-MCA-hydrolysing activity could be recovered, as shown in Fig. 6. A single protein band corresponding to a molecular mass of 60 kDa reacted with the anti-S4 antibody. This indicates that MISP I contains a protein component which is similar to one of the ATPase subunits of the cellular 26S protease complex.

DISCUSSION

The data presented in this paper demonstrate the partial purification of a new ATP-dependent protease from the intermembrane space of rat liver mitochondria, called MISP I. The activity of the enzyme can be stimulated up to 4-fold by Mg^{2+}ATP. The protease is an oligomeric enzyme with a molecular mass of 200 kDa. The presently known ATP-dependent proteases of both pro- and eukaryotic cells are multimeric complexes, the molecular weights being 750 kDa for the Clp-protease of *E. coli* (*33*), 700 kDa for *Hsl*V-*Hsl*U of *E. coli* (*34*), 450 kDa for the bacterial Lon protease (*35*) and its homolog in the matrix of mitochondria (*13, 16*), 850 kDa for the metalloprotease YTA10-12 complex in the yeast mitochondria inner membrane (*23*), and about 2,000 kDa for the 26S protease (*36*). The ATP-dependent protease of the mitochondrial intermembrane space is the smallest ATP-dependent proteolytic complex known to date.

As found in protease inhibition studies, MISP I is a typical thiol protease. In contrast, the bacterial Lon protease (*35*) and its homolog in the matrix of mitochondria (*14, 17*), as well as the bacterial Clp protease (*33*) are serine proteases, whereas the ATP-dependent proteases of the inner membrane of yeast are chelator-sensitive metalloproteases (*20, 22*). The newly discovered ATP-dependent protease, HslV-HslU, of *E. coli*, a proteasome-related protease, is an N-terminal threonine protease (*34*).

MISP I hydrolyses a broad spectrum of synthetic peptide substrates and is therefore a protease with multicatalytic properties or a protease with low substrate specificity. The only known proteolytic enzyme with comparable multicatalytic properties is the cytosolic proteasome or its ATP-dependent form, the 26S protease (*36*). Both proteases, MISP I and the 26S protease, exhibit chymotrypsin-like, trypsin-like, and peptidyl-glutamyl-hydrolysing activities. In contrast to that of the 26S protease the trypsin-like activity of MISP I is not enhanced by ATP.

ATP-binding of MISP I is characterised by a K_m of 200 μM with Suc-LLVY-MCA as the substrate. This is a remarkably high K_m value for ATP-binding compared to the K_m of 30 μM for the ATP-dependent matrix protease of mitochondria (*13*). However, when comparing their ATP affinities one has to take into account that in the case of the matrix enzyme, the determination of the K_m for ATP was performed using different substrates and thus one can not exclude substrate-dependent nucleotide specificity for MISP I (*16*). Mg^{2+}ATP can be replaced by other energy-

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rich nucleotides in the presence of Mg^{2+}, while ADP, AMP, and the non-hydrolysable ATP-analog, β,γ-CH_2-ATP, are not able to promote peptide degradation. The results indicate the requirement of ATP-hydrolysis for ATP-dependent peptide degradation. This was supported by the fact that ADP inhibits the ATP-stimulated peptidase activity. The most effective subsititute for ATP was CTP, followed by UTP, whereas GTP had only a minor effect. These results are similar to those obtained by Goldberg and coworkers for the Lon-protease (*35*) and its homolog in the mitochondrial matrix (*14*), where GTP as well as various nucleoside di- and monophosphates were not effective. MISP I is able to degrade radiolabeled model proteins, although it seems that ATP is not necessary for this protein hydrolysis. This is opposite to in the case of the most well-known ATP-dependent protease, *e.g.* the Lon protease requires ATP for protein, but not for peptide hydrolysis (*37*). The cytosolic 26S protease (*38*), the protease of the inner membrane of yeast mitochondria (*20*), the ATP-dependent matrix protease in rat liver mitochondria (*14*), and the bacterial Lon and Clp proteases (*37, 39*) need ATP for the proteolysis of large protein substrates. For the Clp protease, ATP is essential for the degradation of casein or other protein substrates (*40*), and can not be replaced by other nucleotide triphosphates (*41*). Watabe and Kimura showed that ATP-hydrolysis is not necessarily required for the proteolytic activity of the ATP-dependent protease from the mitochondrial matrix of the adrenal cortex (*15, 16*), and that the nucleotide specificity is dependent on the substrate. Using the peptide substrate, Suc-LLVY-MCA, Hough *et al.* demonstrated the possible replacement of ATP by other energy-rich nucleotides for the 26S protease in the order of ATP>CTP>GTP>UTP (*38*), which is different compared to the data obtained for MISP I, and for the Lon protease (*35*) and its homolog in the mitochondrial matrix (*14*).

Interestingly, MISP I showed immunoreactivity with an antibody raised against ATP binding subunit S4 of the 26S protease complex. The S4 protein is one of the constituents of the PA700 regulatory complex of the 26S protease and is a member of a family of highly conserved ATPases—the AAA family. These ATPases are involved in a variety of cellular processes (*25*). Preliminary peptide sequence analysis of MISP I revealed a peptide exhibiting 80% identity to an AAA-ATPase isolated from plants (Genbank accession number, X74426). Thus, it appears that MISP I is composed of a catalytic and an ATP-dependent regulatory component. Such a composition is not without precedent, having been described for protease complexes such as the 26S protease (*42*) and the Clp protease of *E. coli* (*43*).

The two peaks of proteolytic activity obtained on DEAE-chromatography showed identical sedimentation profiles in glycerol gradients and identical elution profiles on MonoQ. The fact that the two peak fractions are basically identical in their biochemical and enzymatic characteristics raises the possibility that they represent forms of the same enzyme, perhaps different states with various charges due to phosphorylation of the protease, as described for the proteasome, the core particle of the 26S protease (*44*).

To date, a matrix protease (*13-16*) and an inner membrane ATP-dependent protease (*20, 22*) have been described in mitochondria. Earlier findings in our laboratory demonstrated the presence of an ATP-dependent protease in the mitochondrial inner membrane of rabbit reticulocytes (*45*) and rat liver (*46*). It can not be excluded that the ATP-dependent protease described here and in a previous paper (*26*) is loosely associated with the mitochondrial inner membrane and dissociates from the membrane during preparation.

From the results presented here and a comparison with known ATP-dependent proteases, we conclude that MISP I is a new ATP-dependent protease of mitochondria located in the intermembrane space.

We are grateful to our colleagues, Drs. C. Gordon, E. Kominami and H.-G. Holzhuetter, for the gifts of the anti-human-S4 antibody and the helpful discussions.

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