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Regulation of mitochondrial proteolysis

Selective degradation of inner membrane polypeptides

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The in vitro degradation of respiratory chain polypeptide components by a proteinase associated with the intermembrane space fraction was studied in rat liver mitochondria. Differences in susceptibility to proteolysis were detected by gel analysis after electrophoretic separation of the degraded polypeptides. A 55 kDa subunit is protected from proteolysis by the ATP molecule.

Proteolysis; Mitochondria; Polypeptide degradation

1. INTRODUCTION

Regulation of protein turnover is known to take place at all cellular levels [1]. Beyond the physical boundaries of a well delimited organelle such as the mitochondrion, it is foreseeable that a confined proteolytic system takes care of the selective degradation of different proteins. In fact, the proteins from different mitochondrial compartments exhibit a great heterogeneity of half-lives, which implies the existence of regulatory mechanisms for their selective degradation [2]. This regulation can operate at three levels: at the proteolytic enzyme level, at the protein substrate level and through effectors which intervene at the enzyme or the substrate level [3].

A recent report from this laboratory showed that a proteinase with a distinct specificity is localised in the intermembrane space of rat liver mitochondria [4]. The degradation of some of the components of the mitochondrial respiratory chain by the mitochondrial proteinase was studied in vitro. We present evidence that this proteinase is capable of selectively degrading mitochondrial proteins. Furthermore, we have observed that an effector, namely ATP, can modulate this degradation at the protein substrate level.

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2. MATERIALS AND METHODS

2.1. *Preparation of succinate-cytochrome c reductase*

Liver mitochondria were isolated from male Wistar rats [5], suspended at a protein concentration of 40 mg/ml in borate-phosphate buffer (pH 7.8) and disrupted by sonication in an MSC sonifier set at the highest frequency for 2 min with 30 s intervals. The membranes were collected by centrifugation at 160000 × *g* for 60 min and suspended in 0.1 M phosphate buffer (pH 7.4) to a protein concentration of 20 mg/ml. The succinate-cytochrome *c* reductase complex was obtained by sodium cholate solubilization and ammonium sulfate fractionation of the membrane proteins as described by King et al. [6].

2.2. *Protein labelling*

The succinate-cytochrome *c* reductase complex was iodinated with ^{125}I using the chloramine T method [7]. The ^{125}I-labelled proteins had a specific activity of 8000 cpm/μg.

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2.3. *Purification of the intermembrane space proteinase (ISP)*

The intermembrane space fraction was separated by the method of Schnaitman et al. [8] from rat liver mitochondria free of lysosomes [9]. A proteinase producing a characteristic 22 kDa product from κ-casein [4] was partially purified by precipitation of the proteins from the intermembrane space fraction at 60% ammonium sulfate, followed by filtration on Sephadex G-200 and CM-cellulose chromatography (columns equilibrated with 10 mM phosphate buffer, pH 6.0). The proteinase was eluted from the CM-cellulose column with a continuous salt gradient from 0 to 0.25 M KCl.

2.4. *Preparation of lysosomal extracts*

Lysosomes were isolated from the livers of rats injected with Triton WR-1339 as described by Trouet [10].

2.5. *Proteolytic assay*

Protein degradation was determined using two methods: (i) measurement of the acid-soluble radioactive material liberated by hydrolysis of the ^{125}I-labelled polypeptides after incubation with the proteolytic enzymes under the conditions described in the figure legend; (ii) analysis of the degraded polypeptides by electrophoretic separation on a 7–20% gradient polyacrylamide gel (1 mm thick slab) in the presence of SDS [11]. The gels were autoradiographed. Absorbance of the protein bands was scanned at 550 nm along the autoradiogram with a densitometer (Pye Unicam PU8800). The degradation values were determined from the peak areas.

3. RESULTS

A proteinase associated with the mitochondrial intermembrane space (ISP) is able to degrade the succinate-cytochrome *c* reductase complex extracted from the inner membrane. A kinetic study of the overall breakdown of this protein complex shows (fig.1) that it is degraded by the ISP. Hydrolysis proceeds linearly for at least 2 h.

To search for possible differences in the degradation rates amongst the succinate-cytochrome *c* reductase subunits we have analysed the gel electrophoretic patterns as obtained on a gradient polyacrylamide slab gel before and after incubation of the protein complex with the ISP. The labelled polypeptides were identified by autoradiography of the gel (fig.2A). Lane a shows a control where the polypeptides appear discriminated in the gel gradient zone allowing good resolution. The polypeptides under 20 kDa were not distinctly separated due to the impossibility of obtaining a linear relationship at the limit of concentration of the gradient gel. Lanes b–e show the electrophoretic patterns upon degradation by the ISP. The decrease in intensity of the bands in lanes a and c was analysed by densitometry (fig.2B). Decreases of different magnitudes can be clearly detected when the peaks of undegraded and degraded polypeptide bands are compared. Marked differences in the susceptibility to proteolysis exist amongst the polypeptides. Thus the level of the 55 kDa polypeptide decreases by 90% upon degradation while the 26 and 23 kDa polypeptides are only degraded by 10%. This was observed in the presence of Mg^{2+} which increases the overall degradation of the pro-

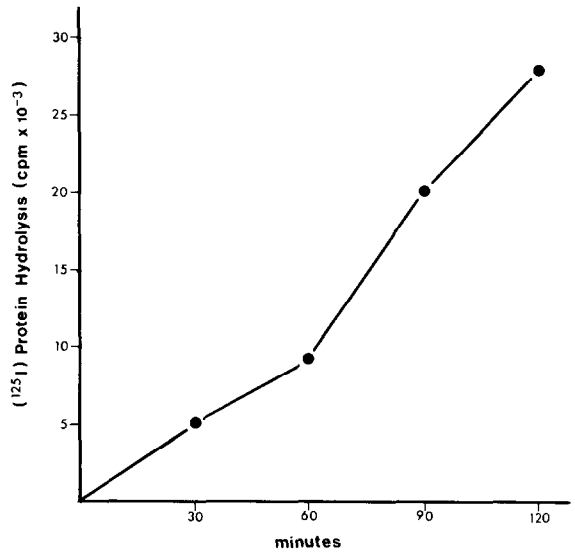

Fig.1. Kinetics of degradation of the succinate-cytochrome *c* reductase complex by the ISP. 50 μl of the reaction mixture (10 mM Tris-HCl, pH 8.5, 2 mM $MgCl_2$, 1.2 μg ISP and 13 μg ^{125}I-labelled succinate-cytochrome *c* reductase) were incubated at 37°C and the reaction stopped by precipitation with 10% cold trichloroacetic acid. After centrifugation the soluble radioactivity was determined in the supernatant.

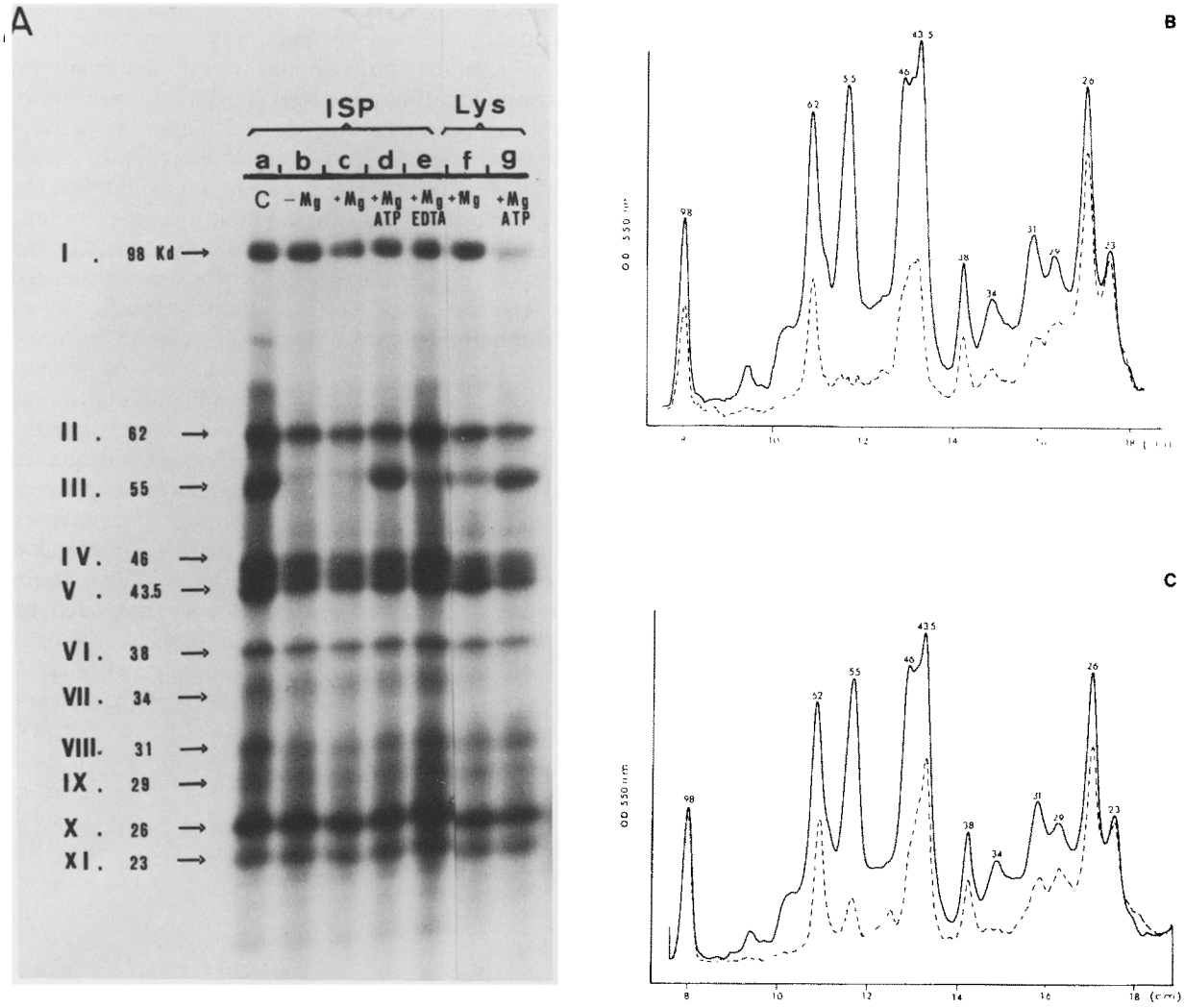

Fig.2. Degradation of the polypeptide components of the succinate-cytochrome *c* reductase complex by the ISP and the lysosomal extract. 20 μg ^{125}I-labelled succinate-cytochrome *c* reductase proteins were incubated with 2.5 μg ISP as described in fig.1 or with 15 μg lysosomal extract under the same conditions. After 4 h incubation at 37°C, the digested polypeptides were separated by SDS-polyacrylamide gel electrophoresis and the gels autoradiographed as described in section 2. (A) Autoradiogram. Lanes: a, control, incubated without enzyme; b, in the absence of Mg^{2+}; c,f, + 2 mM Mg^{2+}; d,g, + 2 mM Mg^{2+} + 2 mM ATP; e, + 2 mM Mg^{2+} + 2 mM EDTA. I–XI indicate main bands of polypeptides. Numbers with arrows refer to molecular mass (in kDa). (B) Densitometric scanning of the autoradiogram on proteolysis by ISP: (——) corresponding to lane a of A; (– – –) corresponding to lane c of A. (C) Densitometric scanning of the autoradiogram on proteolysis by the lysosomal extract: (——) corresponding to lane a of A; (– – –) corresponding to lane f of A. In B and C, peaks are identified by the molecular mass values (in kDa) of the respective polypeptides.

tein bulk (fig.2A, lane c), acting as an activator of the ISP.

To ascertain that the ISP is not of lysosomal nature, this pattern was compared with that obtained by incubating the same substrate, under the same conditions, with a lysosomal extract (fig.2A, lanes f,g). The two patterns present differences (fig.2A–C). The 98, 55 and 44 kDa polypeptides, among others, are clearly less sensitive to lysosomal degradation. These estimates are shown in table 1 which presents the variability of decrease of the different bands in both patterns.

Table 1

Comparative degradation of the polypeptide components of succinate-cytochrome *c* reductase by the mitochondrial ISP and by a lysosomal extract

Band (kDa)	ISP (% decrease)	Lysosomal extract (% decrease)
98	55	5
62	73	42
55	94	71
46, 43.5	65	36
38	58	33
34	56	–
31	64	30
26	19	30
23	12	7

% decrease values calculated from planimetric measure of peak areas of fig.2B,C

ATP does not stimulate the proteolysis of these polypeptides by the ISP; on the contrary, it has a slight inhibitory effect on the overall degradation of the polypeptides, which might be explained by the well known ability of chelating Mg^{2+}. In the case of lysosomal degradation at neutral pH some unexpected stimulation by ATP was observed (fig.2A, lane g, 98 kDa band).

The inhibitory effect of ATP on degradation by the ISP was very striking in the case of the 55 kDa polypeptide. This effect was equally observed in the case of degradation by the lysosomal extract (as shown) and also with a reticulocyte extract (not shown) which we have tested for comparative purposes, since it contains a very active ATP-dependent proteolytic system directed to the destruction of mitochondria during cell maturation [12].

To establish whether the inhibition of proteolysis by ATP was due to a chelation effect, EDTA was introduced in the degradation medium (fig.2A, lane e). In the presence of EDTA overall proteolysis is inhibited and the degradation of the 55 kDa polypeptide is the least affected, suggesting that the strong inhibition of degradation of the polypeptide by ATP (fig.2A, lane d) is not due to Mg^{2+} chelation.

4. DISCUSSION

The present study shows, for the first time, that a mitochondrial proteinase is capable of selectively cleaving subunits of mitochondrial oligomeric structures. A proteinase that we have demonstrated in a previous study [4] to be characteristic of the mitochondrial intermembrane space fraction has a clear degrading activity on the protein components of the mitochondrial respiratory chain. Individual polypeptides of an enzymatic complex, namely succinate-cytochrome *c* reductase, showed different sensitivities to this proteinase. Differences in the vulnerability to proteolytic attack in vitro may actually reflect differences in the inherent susceptibility of proteins to proteases in vivo, since it is known that proteins which turn over rapidly in vivo are also more susceptible to in vitro proteolysis [13]. This general relationship was first observed for soluble proteins or subunits [14]. Membrane proteins are not susceptible to in vitro digestion while they are buried in the membranes of organelles [15]. However, if they are extracted from the membrane complexes their relative sensitivities to commercially available proteases also correlate with their intracellular degradation rates. Hence it is inferred that multimeric proteins are highly dynamic structures which associate and dissociate from the protein-lipid complexes and that their degradation only occurs when they are in a dissociated form [16].

The present data are consistent with the idea that proteolytic enzymes of distinct substrate specificities and different topologies might exist within the mitochondrion in order to assure the selective degradation of proteins in the organelle. Reported work on proteinase degrading yeast mitochondrial translation products [17] and on the turnover of mitochondrial proteins [18,19] corroborate this assumption.

Another important observation concerns the effect of ATP in protecting the 55 kDa polypeptide from proteolysis. This effect seems to take place at the level of the substrate rather than that of the protease since it was equally observed with different proteolytic sources: a mitochondrial protease, a lysosomal extract and a reticulocyte extract.

According to some authors [20,21], the 55 kDa

polypeptide corresponds to one of the major subunits of F_1-ATPase which systematically contaminates the isolated components of complex III of the respiratory chain. This was confirmed under our electrophoretic conditions since the main band of a pure F_1-ATPase [22] co-migrates with the 55 kDa band of our preparation. Since one of these subunits, namely the β, is a binding site for ATP, one could speculate that the observed protection against the degradation of the 55 kDa polypeptide by the ATP represents a regulatory mechanism which works in vivo.

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