PROMINENT ROLE OF LYSOSOMES IN THE PROTEOLYSIS OF RAT LIVER MITOCHONDRIA AT NEUTRAL pH

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1. Introduction

It is known that mitochondrial preparations show marked proteolysis [1]. Although the role of lysosomes in intracellular protein degradation has often been suggested, their direct participation remains to be demonstrated. In as much as mitochondrial preparations contain lysosomes, it is important to find out whether mitoplasts, which should contain a much lower proportion of lysosomes, retain marked proteolytic activity. As shown here, mitoplasts possess much less proteolytic activity than mitochondrial preparations. Moreover, the *N*-acetylglutamate-dependent carbamyl phosphate synthetase (CPS), which is present in the matrix of rat liver mitochondria, is inactivated by macromolecular components present in the digitonin-soluble compartment. Also, separation of mitochondrial populations yield higher proteolytic activity and faster CPS inactivation in the 'light' mitochondria, which contain a much higher proportion of lysosomes, than in the 'heavy' mitochondria. Thus, it appears that lysosomes are responsible for the larger portion if not all protein degradation of mitochondria.

2. Materials and methods

Rat liver mitochondria were obtained and fractionated with digitonin [2]. The 'soluble' fraction (containing the outer membrane and the intermembrane space plus contaminating organelles, including lysosomes) was concentrated with dry Sephadex G-25 coarse. Digitonin was then removed by gel-filtration on Sephadex G-25 superfine, using buffer A or B (see below). Fractions containing the protein were pooled and concentrated with Sephadex to 70–80 mg protein/ml. The mitoplasts, in the terminology of Schnaitman, were obtained in the pellet remaining after 20 min centrifugation at 10 000 × *g* of the mitochondria extracted with digitonin [2]. Heavy and light mitochondria were prepared as described by de Duve et al. [3].

Mitochondria and mitoplasts were washed with buffer A (70 mM sucrose, 220 mM D-mannitol, 5 mM K-phosphate, 5 mM mercaptoethanol (MSH), pH 7.4) and suspended in the buffer to ~100 mg protein/ml. Mitochondria, usually 100 mg protein/ml and mitoplasts were disrupted by suspension in buffer B (5 mM K-phosphate, 5 mM MSH, pH 7.4), followed by freezing and thawing 10 times using a dry ice/acetone bath.

Unless otherwise specified incubations were at 37°C and carried out immediately after preparation of mitochondria or fractions thereof. At the indicated times, a portion was taken and diluted 1:5 in cold 5 mM MSH. One-half was then precipitated with 10% trichloroacetic acid, and protein and ninhydrin-positive material were measured in the precipitate and supernatant, respectively. The other half was centrifuged at 46 000 × *g* for 10 min. Enzyme activities were assayed in the supernatant.

CPS was measured spectrophotometrically [4]. Monoamine oxidase and *N*-acetyl β-glucosaminidase (NAβGase) were assayed essentially as described by Schnaitman et al. [2] and Findlay et al. [5], respectively. Protein was assayed by the biuret method on 10% trichloroacetic acid precipitates (dissolved in

0.2 N KOH). The biuret-deoxycholate technique [6] was used to ascertain the concentration of mitochondria. Bovine serum albumin (fraction V, Sigma Chemical Co.) was used as a standard. Ninhydrin-positive material was assayed as described by Spies [7]. Unless specified, the results are expressed as μmol amino acid (using leucine as a standard) liberated/mg starting protein.

3. Results and discussion

As illustrated in fig.1, there is much more proteolysis at pH 7.4 with mitochondria than with mitoplasts. This finding could be interpreted as due to the removal by digitonin of proteins highly susceptible to proteases located in the mitoplast. However, the proteolysis exhibited by mitochondria and by mitoplasts was roughly proportional to the activity of the lysosomal marker, NAβGase. Moreover, as shown in fig.2, CPS (which comprises ~20% of the mitoplast protein [8]) is inactivated more rapidly in mitochondria, suggesting also that the bulk of proteases has been extracted by digitonin.

Moreover, addition of the digitonin-soluble fraction to mitoplasts restores rapid inactivation. This is likely an enzymatic effect because, as illustrated, it

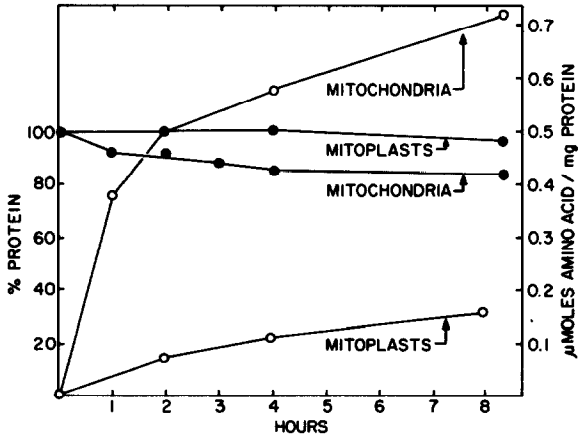

Fig.1. The effect of time on the proteolysis of disrupted mitochondria or mitoplasts. 80 mg protein/ml disrupted mitochondria or mitoplasts in buffer B were incubated. Open circles, acid-soluble ninhydrin-positive material. Closed circles, protein in the acid precipitates expressed as % present at 0-time.

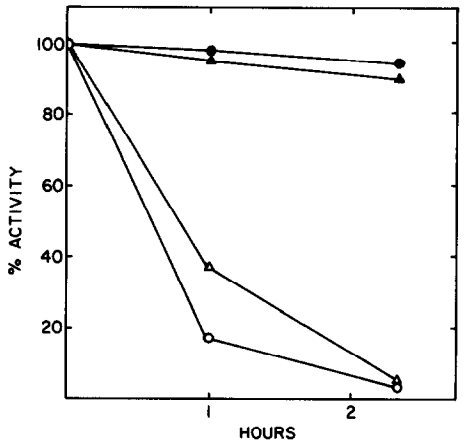

Fig.2. Decay with time of CPS activity in mitochondria, mitoplasts and mitoplasts containing digitonin-soluble portion. All incubations were carried out in buffer B. (○——○) Disrupted mitochondria. 100 mg protein/ml. (●——●) Disrupted mitoplasts, 50 mg protein/ml. (△——△) Disrupted mitoplasts plus digitonin-soluble fraction, 32 mg protein/ml. (▲——▲) Same as open triangles, but using heated digitoninsoluble fraction (boiled for 5 min).

disappears when boiled digitonin fraction is used. And that this reflects proteolysis rather than simply enzyme inactivation is depicted in fig.3, which shows again low proteolysis by mitoplasts and its increase on addition of the digitonin-soluble fraction.

As illustrated in table 1, mitochondria disrupted

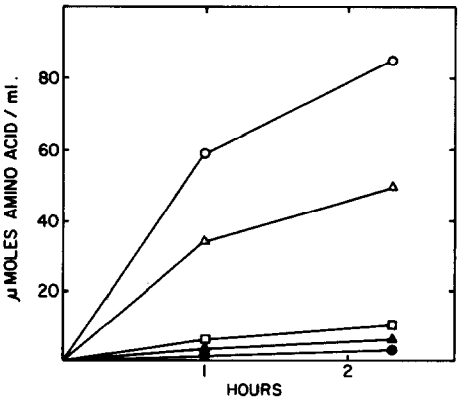

Fig.3. Proteolysis with time of mitochondria, mitoplasts, digitonin-soluble portion and mixtures of this with mitoplasts. The conditions and symbols were as per the legend of fig.2, except that (□—□) corresponds to digitonin-soluble fraction, 32 mg protein/ml and acid-soluble ninhydrin-positive material was measured.

Table 1
Effect of disruption on proteolysis and on stability of CPS in mitochondria and in mixtures of mitoplasts and digitonin-soluble fraction

Preparation	Incubation time (h)	CPS Activity (%)	Amino acid released (μmol/mg protein)
Mitochondria (intact)	2	65	0.44
Mitochondria (disrupted)	2	3	1.
Mitoplast (intact)	1	97	0.24
Mitoplast (disrupted)	1	20	0.47

Mitochondria (100 mg protein) or a mixture of mitoplasts (48 mg protein) and digitonin-soluble fraction (38 mg protein) were used when indicated, in 1 ml of buffer A. When using disrupted preparations, 150 mg protein/ml in buffer B were frozen and thawed (see Materials and methods) and then diluted 1:5-times with the necessary components to yield the final concentrations of buffer A. CPS was measured in intact mitochondria and mitoplasts as follows: 0.2 ml were taken at 0- and at the indicated times, diluted 1:5 with buffer A and centrifuged at 46 000 × *g* for 10 min. The precipitate was suspended to the original volume in buffer B and frozen and thawed 10-times in a dry ice/acetone bath, diluted 1:5 with 5 mM MSH and centrifuged at 46 000 × *g* for 10 min. The activity was measured in the supernatants. Equal activities were obtained at 0-time with intact and disrupted preparations. Other details are given in Materials and methods.

by freezing and thawing had higher proteolytic activity and inactivated CPS faster than 'intact' mitochondria. Moreover, the table shows that the proteases of the digitonin-soluble fraction attack disrupted mitoplasts faster, indicating that the inner mitochondrial membrane protects matrix proteins.

The experiments presented in fig.4 show that at equal protein concentrations proteolysis in 'light' mitochondria is much more marked than in 'heavy' mitochondria and that CPS was inactivated faster by the 'light' mitochondria (which were richer in lysosomes as demonstrated by the higher activity of the lysosomal marker, NAβGase).

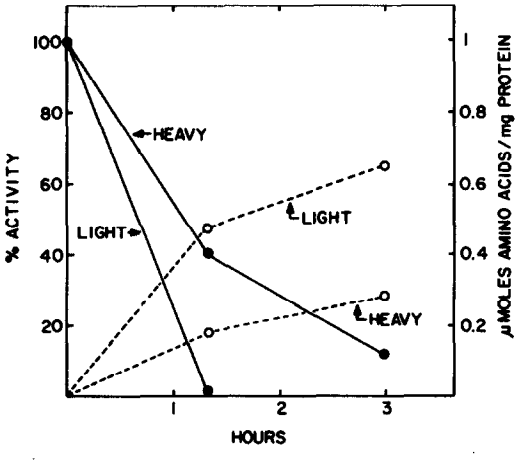

Fig.4. Proteolysis and decrease of CPS activity in preparations of disrupted 'heavy' and 'light' mitochondria. Incubations were at 35°C in buffer B using 100 mg protein/ml. (o- - -o) Acid-soluble ninhydrin-positive material. (●——●) % CPS activity.

Although there have been a number of reports indicating marked proteolysis at neutral pH in mitochondrial preparations [1], lysosomal involvement, while considered, has remained unclear mainly due to technical difficulties in preparing mitochondria free of lysosomes. Our results prove that most of the neutral protease activity of mitochondrial preparations is present in the digitonin-soluble compartment (outer membrane, intermembrane space and contaminating organelles, such as lysosomes). They also strongly suggest that lysosomes are responsible for the bulk of neutral proteolytic activity shown by mitochondria.

Acknowledgements

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