EVIDENCE FOR A PROTEOLYTIC SYSTEM IN RAT LIVER MITOCHONDRIA

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1. Introduction

Analyses of the kinetics of different catabolic processes have revealed that two different types of degradative reactions exist, i.e. a continuous and a discontinuous degradative process [1]. It is likely that a large part of the turnover of liver proteins is a discontinuous process and associated with the turnover of subcellular organelles.

1 Endogenous protein can meet its final fate either in a 'gross degradation' performed by the lysosomes, or in a selective degradation outside the protective membrane of the lysosome. The result of the lysosomal action is an indiscriminate degradation of whole cells or cell compartments, and the organelle has no possibilities to perform the specific regulation of protein degradation which is necessary in the household of the cell. It will thus be evident that the selective regulation of the intracellular protein catabolism needs a system of proteases located outside the protective membrane of the lysosomes, and such enzymes have been reported in different sub-cellular compartments as peroxisomes [2], the nuclei [3,4], the endoplasmic reticulum [3] and mitochondria [3,5–7].

The early studies of Fletcher and Sanadi [8] indicated that the mitochondria turn over as an entity. On the other hand, more recent studies have suggested that the proteins generally turn over more rapidly than mtDNA, and at different rates. For example δ-aminolevulinate synthetase is renewed in a matter of hours [9], while alanine- and ornithine-aminotransferases have a half life of approx. 1 day [10]. Other protein components of the mitochondrion turn over more slowly, with half lives ranging from 4–6 days [10,11]. Since the life span of the hepatocyte nuc-DNA may be 350 days or more [12], it is probable that at least the major components of the mitochondrion are replaced many times during the life time of the liver cell.

The ultimate fate of the mitochondrion is uncertain. In tissue sections of several mammalian cell types, including liver, lysosomes are sometimes found to contain mitochondria [13]. It is possible that these lysosomes are in the process of digesting old and damaged mitochondria. This activity could acount for the turnover of at least a major part of the mitochondria as a unit, but the quantitative importance of lysosomal activity in mitochondrial turnover still remains unknown.

This paper substantiates the existence of a proteolytic system in rat liver mitochondria, and gives an estimate for the relative importance of the 'gross degradation' and the selective degradation.

2. Methods and materials

Mitochondria were prepared from male rats of the Wistar (Møll) strain. The homogenizations were performed in a 50 ml Potter-Elvehjem homogenizer, the pestle rotating at 465 rpm. Differential centrifugations were performed in a refrigerated Sorvall RC-2B centrifuge equipped with a HB-4 swinging bucket rotor (R_{min} = 4.8 cm, R_{max} = 14.6 cm). The homogenate was spun at 2000 rpm (brake on) for 10 min to remove nuclei and cell debris. The supernatant fraction was centrifugated at 8000 rpm for 10 min (brake on), and the pellet resuspended and re-centrifuged two times to reduce the amount of contaminating lysosomes and peroxisomes. The buffer was 225 mM su-

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crose, 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4. Under these conitions the final pellet contains 80% mitochondria, 10% lysosomes and 4% peroxisomes relative to the amounts in the 2000 rpm supernatant [14]. The final 8000 rpm pellet was further purified by a density gradient centrifugation [15], giving a mitochondrial population free of peroxisomes and lysosomes. The mitochondrial pellet thus obtained was resuspended in the isolation buffer and incubated in a shaking water bath. The proteolytic reaction was stopped by rapid cooling in ethanol/CO_2-ice.

Amino acids were extracted by a four step procedure: (1) Sonication of 40 mg mitochondrial protein in 7 ml 2 M pyridine-acetate (pH 5.2), using the microtip and a current input of 5.5 A for 180 sec (Branson sonifier); (2) high speed centrifugation (105 000 g_{av} for 60 min) to remove sub-mitochondrial particles and aggregated lipoproteins; (3) purification of the supernatant amino acids on Bio-Gel P-4 using 2 M pyridine-acetate as solvent, and (4) vacuum evaporation of the eluate.

The residue was disolved in citrate buffer (0.2 N, pH 2.2) and analyzed on a Biochrom amino acid analyzer using the one column system of Dus et al. [16].

The proteolytic activity is expressed as the sum of liberated amino acids/mg mitochondrial protein.

3. Results and discussion

Purified mitochondria were incubated at 37°C and 25°C, and samples for amino acid analyzes were collected every hour (fig. 1). The results suggest that mitochondria contain a proteolytic system, the activity of which was calculated to be 0.656 nmoles amino acid/mg protein at 37°C. Moreover, the proteolytic activity increased with time, and different explanations for this behaviour may be put forward. If the proteolytic activity is first order with respect to the substrate concentration (which means that the proteolytic enzymes are not saturated with substrate) the observed increase may be due to (1) an elevated formation of susceptible conformations of mitochondrial protein, (2) the existence of a proteolytic system which consists of both endopeptidases and exopeptidases, or (3) a combination of (1) and (2). The first

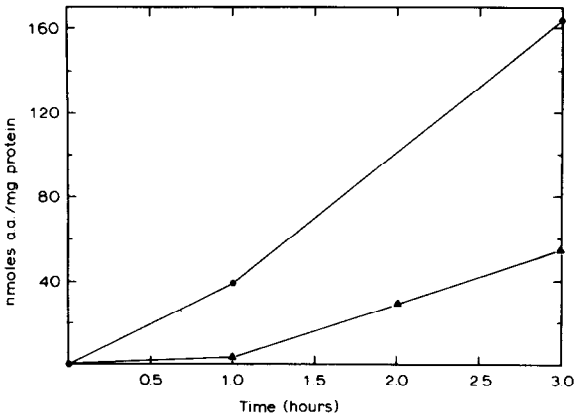

Fig. 1. The proteolytic activity of rat liver mitochondria measured as nmoles free amino acids released per mg of protein as a function of the incubation time at (▲) 25°C and at (●) 37°C.

order rate constant will be 0.104 $days^{-1}$, which corresponds to a half-life of 6.6 days. On the other hand, if the proteolytic activity is zero order with respect to the substrate concentration (which means that the proteolytic enzymes are saturated with substrate), the increased proteolytic activity may be due to an elevated activity of the degradative system. With such kinetics, the rate constant will be 0.104 mg/mg day, and the total life will be 9.6 days.

The first order degradation is in accordance with the present accepted view that the rate of degradation is a reflection of the properties of the substrate molecules, and that the formation of susceptible conformations is the rate limiting step.

The temperature dependence of the proteolytic activity was determined by incubation of 6 identical samples of mitochondria for 2 hr at different temperatures (fig. 2).

An apparent maximum is found at 44°C.

It is now possible to determine the relative importance of the 'gross degradation' caused by lysosomes and the selective degradation caused by the mitochondrial proteolytic system.

The kinetics of the 'gross degradation' indicates a first order process with half-life of 9.4 days [17], as determined by the decay of in vivo labeled mtDNA. The mechanism of lysosomal engulfing can be described in semi-chemical terms:

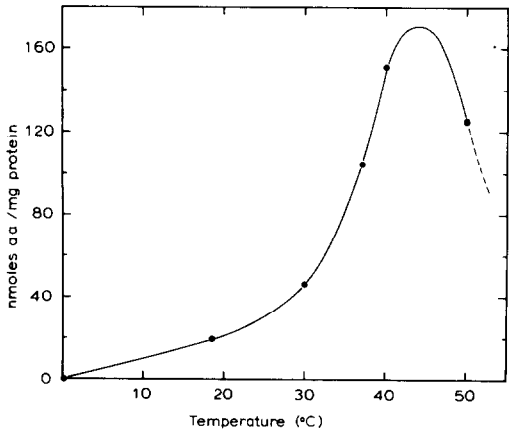

Fig. 2. Effect of the temperature on the mitochondrial proteolytic activity expressed as nmoles free amino acids released per mg of protein during an incubation time of 2 hr.

$$M + L \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ML \xrightarrow{k_2} p \qquad (1)$$

where M = mitochondria; L = lysosomes; ML = mitochondrion–lysosome complex; P = decay products; k_1, k_{-1} and k_2 = rate constants.

Assuming stady state kinetics on the ML complex will by common mathematical procedure give eq. 2:

$$[ML] = \frac{k_1}{k_{-1} + k_2}[M]\,[L] \qquad (2)$$

and the rate constant of the 'gross degradation' will be

$$\frac{d[M]}{dt} = -k_2[ML] = -\frac{k_1 k_2}{k_{-1} + k_2}[M][L] = -k'[M] \qquad (3)$$

if the amount of lysosomes is constant.

Eq. 3 is in accord with the observed kinetics of the decay reaction, with $k' = 0.074$ days^{-1} (equivalent to a half life of 9.4 days for mtDNA).

The selective degradation of mitochondrial proteins can be described in simple terms, assuming that only a limited fraction of the total protein can form susceptible conformations for the mitochondrial proteolytic system.

$$\frac{M'}{M} = f \text{ or } M' = fM \qquad (4)$$

The degradative reaction will be described by eq. 5:

$$M' \xrightarrow{k_3} P \qquad (5)$$

with kinetics described by eq. 6:

$$\frac{d[M']}{dt} = -k_3[M'] = -k_3 f[M] \qquad (6)$$

The total degradation rate will be the sum of the lysosomal degradation, eq. 3, and the selective degradation, eq. 6, and is given by eq. 7:

$$\frac{d[M]}{dt} = -k'[M] - k_2 f[M] = -k[M] \text{ as } k = k' + k_3 f \qquad (7)$$

The rate constant k for the sum degradation has been determined by Arias et al. [18] to be 0.102 days^{-1} (equivalent to a half life of 6.8 days), while the intramitochondrial proteolytic activity has an in vitro rate constant (k_3) of 0.104 days^{-1} at 37°C (this paper).

The fraction of mitochondrial protein forming susceptible conformations for the selective degradation can now be calculated from eqs. 7 and 8:

$$\begin{aligned} k' &= 0.074 \text{ days}^{-1} \\ k_3 &= 0.104 \text{ days}^{-1} \\ k &= 0.102 \text{ days}^{-1} \end{aligned} \qquad f = \frac{k - k'}{k_3} = 0.27 \qquad (8)$$

These calculations indicate that 27% of the mitochondrial proteins are labile with respect to the mitochondrial proteolytic system. Lysosomes will thus play an important role in maintaining a steady state level of mitochondria in the cell, while the intramitochondrial proteases possibly regulate the enzyme levels of the organelle, as well as they are generating a sink for the transfer of extramitochondrially produced protein.

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