ON A NEUTRAL PROTEOLYTIC SYSTEM IN RAT LIVER MITOCHONDRIA

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1. Introduction

Although some evidence has accumulated in recent years indicating the existence of a proteolytic system in rat liver mitochondria [1–4], doubts persist as to whether mitochondrial proteolysis is due to lysosomal hydrolases adsorbed to the mitochondrial outer membrane. After exposure of mitochondria to digitonin, the resulting mitoplasts retain very little of the original capacity of mitochondria for auto-proteolysis [5]. This finding was utilised as a major argument that contaminating lysosomal enzymes are responsible for the proteolytic activity of mitochondria, inasmuch as removal of the outer membrane by digitonin represents the elimination of any such contaminating organelles.

Here we show that the proteolytic activity of mitochondria towards exogenous substrates is differently influenced by digitonin treatment. Whereas the degradation of casein, a non-mitochondrial protein, was drastically reduced if the mitochondria used as source of proteinase were pre-exposed to digitonin, the degradation of mitochondrial substrates such as cytochromes, was affected very little. Moreover studies on the pH dependence of the mitochondrial proteolytic activity have shown that digitonin treatment only eliminates the acid proteolytic activity, while it reveals a marked peak of activity at pH 7.0. These results favour the view that neutral proteolytic activity in mitochondrial fractions that have been exposed to digitonin, represents a proteolytic system of true mitochondrial nature.

2. Experimental

Two month old male Wistar rats were fasted overnight and decapitated immediately before the experiment. The livers were perfused by injection with saline into the portal vein.

Mitochondria were prepared according to [6] or [7]. In the latter method, mitochondrial suspensions were treated for 20 min at 0°C with digitonin (5 μg/mg mitochondrial protein). Mitochondria (treated or untreated) were then submitted to osmotic shock [8] and centrifuged at 105 000 × *g* for 1 h. The 105 000 × *g* supernatant (soluble extract of crude mitochondria) was used as enzyme source for the proteolytic assays.

A fraction containing cytochromes was isolated from mitochondria by the method in [9]. These cytochromes were iodinated with ^{125}I using the chloramine T method [10]. ^{125}I-Labelled casein was kindly provided by Dr Ph. Regnier (Institut de Biologie Physico-Chimique, Paris).

Proteins were estimated by the Lowry method [11]; LAB-TROL from Dade Co., Miami, FL, was taken as standard.

The proteolytic activity was determined by the hydrolysis of ^{125}I-labelled proteins. The reaction mixture contained in 120 μl total vol.: 6 μmol phosphate buffer (pH 7.0), 120 μg cytochromes (of which 30 μg where ^{125}I-labelled with spec. act. 2000 cpm/μg) or 140 μg casein (of which 50 μg were ^{125}I-labelled with spec. act. 1400 cpm/μg) and 40–80 μg of the mitochondrial soluble extract. The same proportion of labelled to cold substrates was maintained throughout all the experiments, since we observed that iodinated and unlabelled proteins are degraded at different rates. The incubation time was 60 min at 37°C and the reaction was stopped by addition of 30 μl of trichloroacetic acid 50%. After 30 min at 0°C the precipitate was removed by centrifugation and the acid soluble radioactivity measured in a gamma counter (Picker-Pace 1).

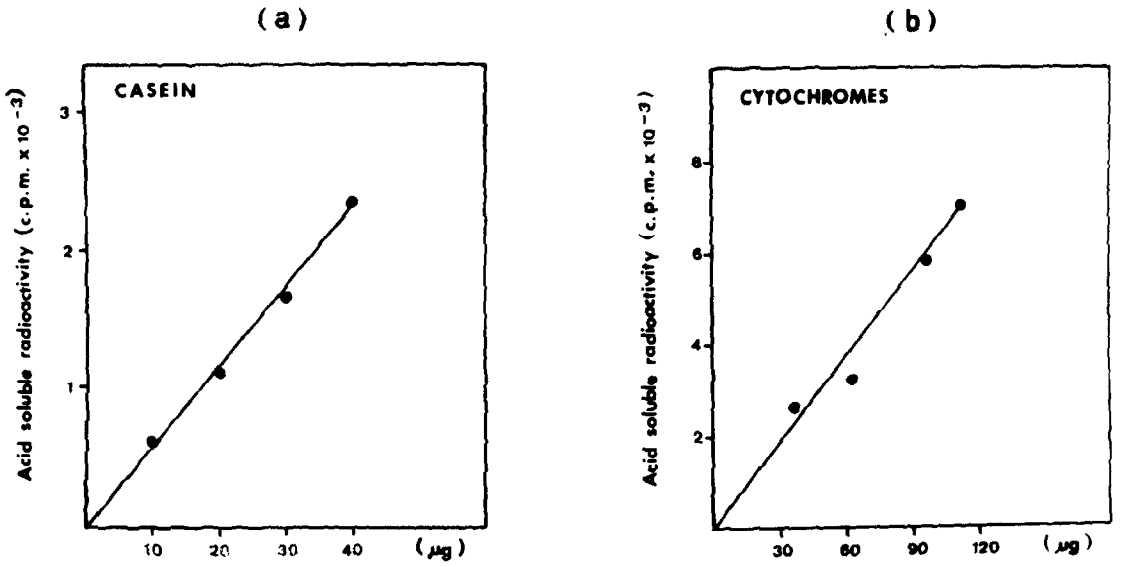


Fig.1. Hydrolysis of ^{125}I-labelled substrates by the soluble extract of untreated mitochondria. Experiments (a) and (b) were carried out, respectively, with casein and cytochromes. See section 2 for additional information.

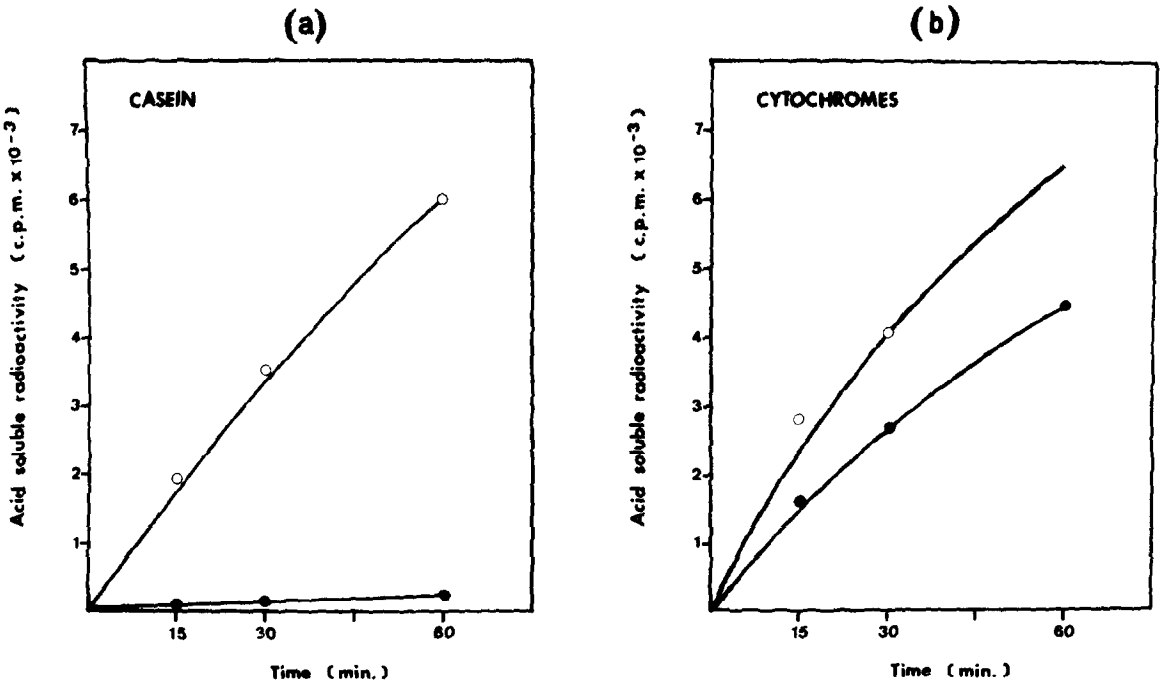


Fig.2. Kinetics of hydrolysis of ^{125}I-labelled proteins, (a) casein and (b) mitochondrial cytochromes, by the soluble extract from untreated mitochondria (○——○) and digitonin treated mitochondria (●——●). Conditions as described in section 2. Values are the mean of triplicates from 2 different experiments.

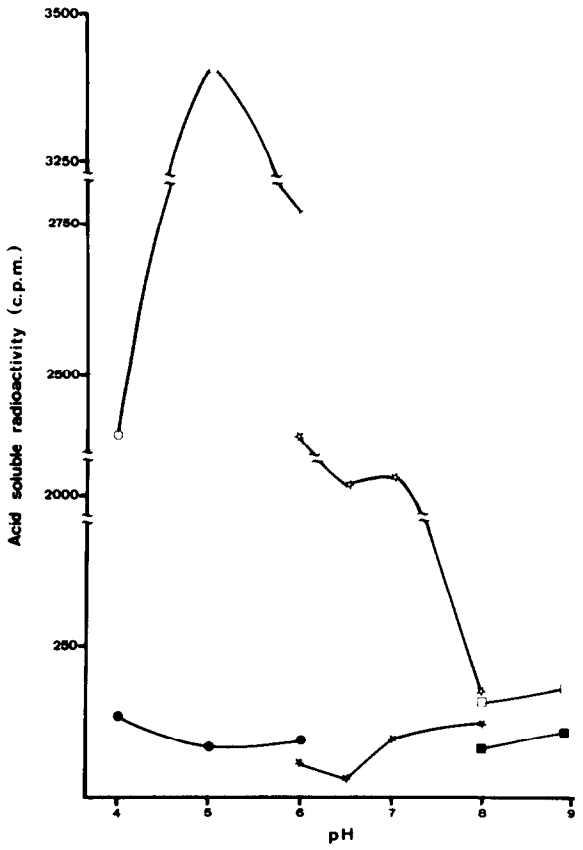
Fig.3. Effect of the pH on the degradation of ^{125}I-labelled casein by the soluble extract of untreated mitochondria (open symbols) and digitonin treated mitochondria (black symbols). The incubation mixtures were made with the following buffers (0.05 M): (○) citric acid–citrate; (☆) potassium phosphate and (□) Tris–HCl. Values are the mean from 2 determinations in a representative experiment.

3. Results

Mitochondrial cytochromes and a non-mitochondrial protein, casein, were utilised as substrates for proteolytic assays. The hydrolysis of these substrates was proportional to the amount of enzyme extract added, as shown in fig.1.

Figure 2 depicts, for each substrate, the relative kinetics of proteolysis if the protease source is the soluble extract from digitonin treated instead of untreated mitochondria. The caseinolytic activity is virtually abolished which indeed suggests an involvement of contaminating hydrolases in this degradation. The proteolytic activities towards the mitochondrial cytochromes are much less affected by the digitonin treatment of the mitochondria used as source of proteases: a reduction of 33% is observed, which could be attributable to contaminating enzymes. Still, the remaining proteolytic activities are considerable and must be of true mitochondrial nature, since digitonin depleted the mitochondria of contaminating lysosomes. In fact, < 14% of the initial acid phosphatase activity remained in the final mitochondrial preparation after digitonin treatment (not shown).

After digitonin treatment the mitochondrial proteolytic activity is lost mainly over the acid pH range. Towards casein the remaining activity becomes negligible over pH 4–9 (fig.3). Towards the mitochondrial cytochromes, although digitonin treatment results in a comparable loss of the acid proteolytic activity, it reveals a clear peak at neutral pH (fig.4).



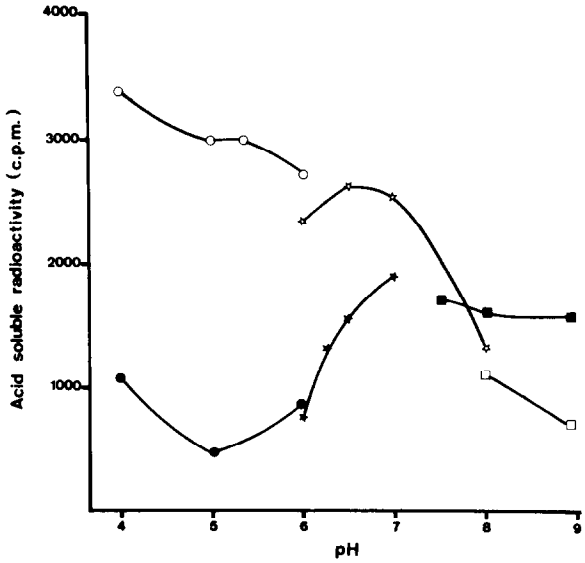


Fig.4. Effect of the pH on the degradation of ^{125}I-labelled cytochromes by the soluble extract of mitochondria (open symbols) and digitonin-treated mitochondria (black symbols). Conditions as in fig.3.

4. Discussion

The present experiments suggest the existence of a proteolytic system in rat liver mitochondria, capable of degrading mitochondrial cytochromes. This proteolytic activity was recovered in the mitochondrial crude extract in a soluble form; however, the possibility remains that the hypotonic lysis which the mitochondria underwent might have solubilized proteases from the membrane structures.

Prior perfusion of the livers prevents contamination of the mitochondrial preparation with blood enzymes. To exclude the possibility that the proteases might be due to contaminating lysosomes, we submitted the mitochondrial fraction to a digitonin treatment. The digitonin concentration utilised was not sufficient to remove the outer membrane of the mitochondria, since it is important, for our purposes, not to affect the integrity of the organelle.

Striking differences were observed with digitonin treated mitochondria depending on whether mitochondrial cytochromes or casein were used as substrate: it is remarkable that hydrolysis of the cytochromes is much less affected by such a treatment than hydrolysis of a non-mitochondrial substrate such as casein, which is practically abolished.

According to the extraction procedure [9] the cytochrome fraction reported here contains cytochromes *a*, *b* and c_1. In [12] we also observed a proteolytic activity in mitochondria solubilized by Triton X-100, towards cytochrome *c* (horse heart, Sigma); this activity seems to be the same as the one presently described. It must differ from the membrane-bound proteinase detected in rat liver mitochondria [13] since the latter enzyme is not active towards cytochrome *c*.

It is premature to suggest a role for the mitochondrial proteolytic system described here. Proteolytic enzymes undoubtedly must exist in particular structures to carry out specific degradations related to the regulation of enzyme levels or to the turnover of proteins, but only further work can establish whether the proteolytic activity reported here performs that regulatory function.

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