Study on the localization of proteases of mitochondrial origin.

M. C. DUQUE-MAGALHAES † * and Philippe REGNIER **.

(Reçu le 11-12-1981, accepté après révision le 2-6-82).

Résumé.

Une forte activité protéolytique peut être mise en évidence dans les mitochondries de foie de rat en utilisant la caséine comme substrat. Les protéases qui dégradent la caséine sont réparties dans la fraction sédimentable (Po) et dans l’extrait soluble (So). Une partie de l’activité de la fraction soluble que l’on retrouve dans l’espace intermembranaire provient d’une contamination par des protéases lysosomales qui peuvent être éliminées en lavant les mitochondries à la digitonine. Ce lavage a pour effet d’augmenter l’activité caséinolytique associée aux fragments de membrane ce qui démontre que celle-ci n’est pas due à des enzymes lysosomaux. Lorsque les rats subissent des injections du produit 48/80 qui, en dégranulant les mastocytes empêchent la contamination des préparations de mitochondries par les protéases mastocytaires, la fraction membranaire (Po) possède une activité caséinolytique qui représente 80 p. cent de l’activité des préparations témoins. Une activité de même ordre est associée aux membranes de mitochondries de cerveau de rat débarrassées des rares mastocytes qu’elles contiennent. La plus grande partie de l’activité caséinolytique associée aux membranes de foie de rat n’est donc pas d’origine mastocytaire. Les mitochondries débarrassées de contaminations lysosomaux par la digitonine ont été fractionnées de façon à séparer la matrice, la membrane interne, l’espace intermembranaire et la membrane externe. Les quatre fractions possèdent une activité protéolytique. Cependant la plus grande partie de celle-ci est associée aux compartiments de l’intérieur de la mitochondrie : la matrice et la membrane interne. Le pH optimum et la sensibilité aux inhibiteurs des protéases des différents compartiments indiquent que ce sont des enzymes différentes.

Mots-clés : protéases / mitochondries / localisation / origine cellulaire.

Summary.

A marked proteolytic activity against casein can be demonstrated in rat liver mitochondria. The proteases degrading casein appear distributed between a sedimentable fraction (Po) and a soluble extract (So). Part of the soluble fraction activity, which may be recovered in the mitochondrial intermembrane space, results from a contamination by lysosomal proteases and can be eliminated by previously washing the mitochondria with digitonin. The pre-exposure to digitonin causes an enhancement of the caseinolytic activity associated with the membrane fragments, proving that this activity is not due to lysosomal enzymes. When rats have been injected in vivo with the compound 48/80 which, by degranulating the mast cells prevents contamination of the mitochondrial preparations by mast cell proteases, the membrane fraction (Po) retains a caseinolytic activity of the order of 80 per cent of the control preparations. A similar value of activity is observed in the membranes of brain mitochondria, isolated by a method which removes the rare mast cells they may contain. This shows that the greater part of the caseinolytic activity associated with the liver membranes does not originate from mast cell granules. Liver mitochondria pre-exposed to digitonin to eliminate lysosomal contaminants, have been subfractionated into matrix, intermembrane space, inner and outer membrane. Each of the fractions exhibits a caseinolytic activity, but the largest part is localized in the inner compartments of mitochondria: the matrix and the inner membrane. The optimal pH and the sensitivity to inhibitors of the proteases in the different compartments indicate that we are dealing with distinct enzymes.

Key-words : proteases / mitochondria / localization / cell origin.

† To whom all correspondence should be addressed.
Introduction.

A number of published results describing both soluble [1-4] and membrane associated [5-9] proteases in mitochondria, have substantiated the concept that these organelles have their own protein degrading system; this was in some way previsible since they also have a protein synthetic system. The fact that some mitochondrial proteins, synthesized in the cytoplasm as larger precursors, are processed by limited proteolysis during the transport into mitochondria [10], has corroborated the existence of mitochondrial proteases. It is difficult however to prove their true identity with the organelles. Indeed lysosomal contamination has been made responsible for the soluble activity [11], while mast cell enzymes seem to be at the origin of the membrane associated one [12].

In a previous work [13] we had examined the involvement of lysosomal enzymes in the caseinolytic activity of the different mitochondrial fractions. The present report reevaluates the precise mitochondrial localization of this caseinolytic activity and moreover investigates the origin of the particulate enzyme responsible for the activity associated with the mitochondrial membranes. We have attempted a preliminary characterization of these caseinolytic activities in the different mitochondrial compartments.

Material and Methods.

Chemicals.

All chemicals were of analytical grade. $^{125}$I iodide was from Amersham Radiochemical Centre.

Preparation of mitochondria and submitochondrial fractions.

Mitochondria were isolated from male Wistar rat livers, by the differential centrifugation method of Loewenstein [14] which purifies mitochondria by washing with low concentrations of digitonin (1.75 mg digitonin per g original liver). In control preparations the digitonin washing step was replaced by isolation medium.

Mitochondria (untreated or digitonin treated) were then submitted to osmotic shock in water, 30 min at 0°C, followed by a 144,000 g centrifugation for 1 h. Both the pellet (Po) and the supernatant (So) were used as enzymatic sources for the proteolytic assay.

In other experiments, mitochondria were isolated by a modification to the method of Loewenstein which consists in using 1.0 mg instead of 1.75 mg digitonin per g original liver. The purified mitochondria were fractionated in matrix, inner membrane, intermembrane space and outer membrane, by the procedure of Schnaitmann [15] using 0.12 mg digitonin per mg mitochondrial protein to solubilize the outer membrane, which is separated by a 12,000 g centrifugation, and further disrupting the inner membrane by sonication at 20 KHz (3 × 30 s).

The mitochondrial fractions were kept in small aliquots at — 70°C.

Preparation of lysosomal fractions.

Lysosomes were isolated from liver of rats injected with Triton WR-1339 [16].

Experiments with 48/80.

To deplete the mast cells of their granules, rats were injected i.p. with increasing doses of the compound 48/80 (a polymer of N-methylomanoisylamine and formaldehyde) for five days. Each rat received 100 μg/100 g b.w. on the first day, 200 μg/100 g b.w. on the second day and increasing doses in this proportion up to 500 μg/100 g b.w. on the fifth day. The animals were killed on the sixth day, after a 24 h starvation period. The fractionation procedures to obtain the mitochondrial components were as described above.

Preparation of mitochondria from rat brains.

A method using a sucrose gradient to separate the synaptic vesicles and myelin from the mitochondria [17] enable isolation of mitochondria without mast cells. The intact mitochondria were purified by washing with low concentrations of digitonin and fractionated in a soluble and a membrane fraction by the same method already described for liver.

Casein labelling.

Casein (Hammarsten, BDH chemicals) was iodinated with $^{125}$I (Amersham, England) using the chloramine T method [18].

Proteolytic assay.

The proteolytic activity was determined by hydrolysis of $^{125}$I-labelled casein. The reaction mixture contained in 120 μl total volume : 6 μmoles phosphate buffer pH 7.5, (or other buffers at other pH values for the study of pH effect), 40 μg $^{125}$I casein (from which 10 μg were $^{125}$I-labelled with sp. act. 9000 cpm/μg) and 90 μg enzymatic protein. The incubation was for 60 min at 37°C and the reaction was stopped by the addition of 30 μl of TCA 50 per cent. 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).

Analysis of the digestion products.

The 24 h digestion products in the incubation medium without TCA addition, were analyzed by ascending paper chromatography (Whatman paper, no. 1), using acetic acid, N-butanol, water, 10:78:12.

Sodium iodide moniodotyrosine and diiodotyrosine were used as standards. The chromatograms were deve-
Localization of proteases of mitochondrial origin.

lopped with palladium, chloride and ninhydrin. The distribution of radioactivity in the chromatogram was determined by counting 1-cm-wide strips of the chromatographic paper in the gamma counter.

Other assays.

Marker enzymes in mitochondrial subfractions were assayed by the following methods: malate dehydrogenase [19], succinate dehydrogenase [20], adenylate kinase [21], monoamine oxidase [22], acid phosphatase [23], and cathepsin D [24].

The proteins were determined by the method of Lowry [25] using LAB-TROL as a standard.

Results.

Identification of solubilized $^{125}$I as casein degradation products.

Since the hydrolysis of $^{125}$I casein was estimated by the radioactivity of the acid soluble peptides obtained after precipitation of non digested casein, it was essential to prove that the acid soluble radioactivity truly reflected the extent of proteolytic activity. Although unspecific dehalogenation was always corrected for by appropriate blanks, the possibility remained that an enzymatic dehalogenation could be responsible for the acid soluble radioactivity. Analysis by ascending paper chromatography of $^{125}$I-labelled casein which had been digested 24 h with the mitochondrial membrane fraction Po, showed that the major degradation product was monoiodotyrosine.

Localization of caseinolytic activity in soluble and pelleted mitochondrial subfractions.

When digitonin treated mitochondria are suspended in water for osmotic shock and centrifuged at 144,000 g, a pellet (Po) and a supernatant (So) are obtained. As estimated from the repartition of marker enzymes, the pellet contains the two mitochondrial membranes and a large part of the matrix proteins, while the supernatant contains the intermembrane space and the remaining of the matrix fraction. The proteolytic activity distribution is 70 per cent in the pelleted fraction and 30 per cent in the soluble fraction.

Effect of elimination of lysosomal contaminants, on the caseinolytic activity of mitochondrial soluble and pelleted subfractions.

The specific activities of the lysosomal markers, acid phosphatase and cathepsin D, in whole mitochondria and subfractions Po an So, are markedly reduced by washing with digitonin at concentra-

BIOCHIMIE, 1982, 64, n° 10.
brain, a tissue which is practically devoid of mast cells. The average caseinolytic specific activity of control liver mitochondria is 18.5 µg of casein solubilized in one hour by one mg of proteins, in two different experiments. This value is decreased to 15.0 (µg casein/hr.mg protein\(^{-1}\)) upon treatment with the compound 48/80. The caseinolytic specific activity of mitochondrial membranes isolated from rat brain is 13.4 µg casein/hr.mg protein\(^{-1}\).

**Submitochondrial localization of the caseinolytic activity.**

To further investigate the intramitochondrial localization of the caseinolytic enzymes, the activities were determined in mitochondrial subfractions of more precise derivation. Before subfractionation, the isolated mitochondria were purified by exposure to low concentrations of digitonin that solubilizes most of the contaminating lysosomal enzymes. The digitonin concentration had to be reduced from the value of 1.75 mg per g original liver, recommended in a standard method [14], down to 1.0 mg per g original liver, in order to avoid an impairment of the ulterior subfractionation in mitochondrial compartments. This concentration of digitonin is sufficient to reduce by 90 per cent the lysosomal contamination, as monitored by the enzyme markers, acid phosphatase [13] and cathepsin D (unpublished results). From these purified mitochondria the following subfractions were prepared: matrix, intermembrane space, inner and outer membrane. These fractions were characterized by marker enzymes, namely: malate dehydrogenase, adenylate kinase, succinate dehydrogenase, and monoamine oxidase.

The distribution of the marker enzymes and caseinolytic activity is shown in table I. The matrix fractions represents 32 per cent of the total protein of the mitochondria and contains 66 per cent of its marker enzyme, malate dehydrogenase. The inner membrane fraction which represents 40 per cent of the whole mitochondrial protein, exhibits its marker enzyme, succinate dehydrogenase, in a value that exceeds the total in the intact mitochondria, meaning that the substrate has better accessibility to the subfraction. This inner membrane fraction is heavily contaminated by matrix proteins, as demonstrated by the presence of 23 per cent of malate dehydrogenase activity. The intermembrane space fraction contains almost all of the respective enzyme marker, adenylate kinase, but contains also proteins from the outer and inner membrane fractions. Conversely, the outer membrane fraction is hardly contaminated by the other fractions but its marker enzyme, monoamine oxidase, is poorly recovered at a percentage of only 35 per cent. We tried to reduce this loss of the outer membrane proteins but the need of a purification step, with pre-exposure of mitochondria to low concentrations of digitonin, persistently resulted in impaired separation of the outer membrane fraction.

The caseinolytic activity is mainly recovered with the matrix and the inner membrane fractions, the specific activity being much higher in the inner membrane fraction than in other compartments.

**Preliminary characterization of the caseinolytic activities of the mitochondrial compartments.**

As shown in figure 2 and table II the matrix subfraction presents two peaks of activity at pH 8.0 and 9.5 and is sensitive to trypsin and chymotrypsin-like protease inhibitors, and to NEM and

<table>
<thead>
<tr>
<th>Table I.</th>
<th>Protease distribution in purified mitochondria.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Malate dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>Sp. act.</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>28.8</td>
</tr>
<tr>
<td>Matrix</td>
<td>58.5</td>
</tr>
<tr>
<td>Inner membrane</td>
<td>16.5</td>
</tr>
<tr>
<td>Intermembrane space</td>
<td>3.0</td>
</tr>
<tr>
<td>Outer membrane</td>
<td>2.6</td>
</tr>
</tbody>
</table>

*Data are average values of three separate experiments. The specific activities of the marker enzymes are expressed in Δ O.D. per 10 min per mg protein. Protease activities as µg casein hydrolyzed per hour. Total recoveries of the enzymes as percentages of the values for whole mitochondria.*

*BIOCHIMIE, 1982, 64, n° 10.
Localization of proteases of mitochondrial origin. PCMB. Thus it seems likely that at least two proteases exist in the matrix.

![Diagram of pH dependence of caseinolytic activity](image)

**FIG. 2. — pH dependence of the caseinolytic activity of mitochondrial subfractions.** The buffers were, at 50 mM, sodium phosphate (○), Tris-HCl (△) and glycine-NaOH (□).

The caseinolytic activity in the inner membrane fraction has only one peak of activity at pH 7.8, being less active in Tris-HCl than in phosphate or glycine buffers. This activity is sensitive to both seryl and sulphydryl group reagents and significantly inhibited by EDTA. Part of this inner membrane activity, probably the one which is sensitive to PCMB, might be due to contamination by the matrix proteases (vide table I).

The intermembrane space fraction caseinolytic activity has a maximum at acid pH (6.5). It is weakly inhibited by the methyl ketones, EDTA and PCMB.

The outer membrane fraction clearly contains a thiol protease.

All the preceding data are assembled in figure 2 and table II.

**Discussion.**

Rat liver mitochondria were subfractionated after disruption of the organelle by hypotonic lysis in water, which permitted the ultracentrifugal separation of a soluble extract (So), and a membranous fraction (Po). Although these two subfractions did not perfectly separate the soluble from the membranous compartments of mitochondria, the approach was still useful to provide a first insight on the distribution of a caseinolytic activity within the organelle. In fact, 70 per cent of the enzymes degrading casein appear associated with the pelleted material which consists of all the membranous structures plus a large part of the matrix soluble proteins. The remaining 30 per cent of the proteolytic activity is in the soluble extract.

**Table II.**

<table>
<thead>
<tr>
<th>Addition of</th>
<th>Matrix pH 7.5</th>
<th>Matrix pH 9.5</th>
<th>Inner membrane pH 8</th>
<th>Inner membrane pH 6.5</th>
<th>Inner membrane pH 7.5</th>
<th>Outer membrane pH 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF 1 mM</td>
<td>90</td>
<td>130</td>
<td></td>
<td></td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>DFP 1 mM</td>
<td>53</td>
<td>96</td>
<td></td>
<td></td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>TLCK 5 mM</td>
<td>28</td>
<td>70</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM</td>
<td>65</td>
<td>66</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPCK 1 mM</td>
<td>32</td>
<td>77</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA 1 mM</td>
<td>47</td>
<td>65</td>
<td>83</td>
<td>30</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>NEM 1 mM</td>
<td>39</td>
<td>65</td>
<td>84</td>
<td>30</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>PCMB 1 mM</td>
<td>37</td>
<td>28</td>
<td>84</td>
<td>30</td>
<td>100</td>
<td>70</td>
</tr>
</tbody>
</table>

Effect of inhibitors on the caseinolytic activities of mitochondrial subfractions. Proteolytic activities in each subfraction were measured at the optimal pH as obtained in figure 2.
which represents the totality of the intermembrane space proteins and also a fraction of the matrix.

These experiments were carried out with mitochondrial preparations isolated by a conventional method [14] which uses low concentrations of digitonin in a washing step to solubilize lysosomal enzymes that contaminate mitochondria. The reduction in the total activities of the purified whole mitochondria is totally reflected in the respective soluble extracts. Conversely, the enhancement of the membrane-bound proteolytic activity indicates that this fraction does not contain lysosomal proteases even when they originate from unpurified mitochondria. The question remains of knowing why the membrane-associated activity is enhanced after exposure of the original mitochondria to low concentrations of digitonin. It seems likely that the detergent abolishes the latency of the particulate enzyme by alteration of the membrane structure.

Another important question is raised by previous verifications concerning the mast cell origin of proteases originally thought to be constituents of mitochondria. In fact, a chymotrypsin-like mast cell protease has been isolated from inner membrane mitochondria by using histones [25] and pyridoxal enzymes [26] as substrates. In the present work we used a different substrate: casein, which is not degraded by the mast cell enzyme [26]. Moreover, the caseinolytic activity we observed associated with the mitochondrial inner membrane is only slightly inhibited by DFP and TPCK in contrast with the total inhibition observed in the case of the mast cell enzyme.

We investigated the effect of in vivo disrupting rat mast cells disruption on the caseinolytic activity of mitochondrial membranes. The compound 48/80 is able to degranulate rat mast cells as indicated by histological observation of various rat tissues [28]. This treatment causes only a slight reduction (~20 per cent) of the caseinolytic activity compared with the considerable reduction (~70 per cent) obtained for the mitochondrial associated protease of mast cell origin [12]. The caseinolytic activity remaining after treatment with the drug must essentially originate from mitochondria. An identical level of caseinolysis is found in brain mitochondrial membranes obtained from preparations totally devoid of mast cells [17].

Attempts were made to localize more precisely the caseinolytic activity within rat liver mitochondria. For that purpose, purified mitochondria were subfractionated in four compartments characterized by enzymatic markers. The separation of the outer membrane was impaired when the original mitochondria was purified with the same digitonin concentration used prior to the subfractionation in Po and So after osmotic lysis. As reported elsewhere [13], an effective separation of the outer membrane from purified mitochondria was made possible by using 1.0 instead of 1.75 mg digitonin per g original liver in the purification procedure.

The caseinolytic activity is mainly recovered with the matrix and inner membrane fractions, although the specific activities are also important for the intermembrane space and outer membrane proteins.

The matrix exhibits a marked proteolytic activity which can originate in at least two proteases, one being serine and another thiol in nature. A proteolytic activity is also associated to the inner membrane. Part of this activity could originate in a contamination with the thiol protease from the matrix. The activity which is inhibited by the chymotrypsin inhibitor might reflect the contamination of this fraction by a mast cell protease.

The outer membrane contains a weaker proteolytic activity, due to a thiol enzyme. Inasmuch as this fraction is devoid of contamination by the other fractions, it seems likely that this enzyme is specifically associated to the outer membrane of mitochondria.

The proteolytic activity in the intermembrane space is relatively weak and the results obtained after addition of specific inhibitors do not allow any conclusions indicating a particular enzyme. However, the results obtained in studies on the specificity of the proteases of the different mitochondrial compartments indicate that the intermembrane space fraction contains a protease which does not exist elsewhere in mitochondria (unpublished results).

In general, our results indicate that various proteases exist in liver mitochondrial preparations obtained under conditions which considerably decrease lysosomal contamination. This is in agreement with the finding that the proteases associated with the matrix, the inner membrane and the intermembrane space have different specificities with respect to the neutral protease from lysosomes. Mast cell enzymes could exist but, as shown in control experiments, they would not exceed 20 per cent of the total activity in the particulate fraction.

Acknowledgements.

This work was supported in part by travel funds awarded by the French Ministry of Foreign Affairs. We thank Miss Rosa Maria Santos for her skillful technical assistance.
Localization of proteases of mitochondrial origin.

REFERENCES.