Discrimination of distinct proteinases at the four structural levels of rat liver mitochondria

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Rat liver mitochondrial fractions corresponding to four morphological structures (matrix, inner membrane, intermembrane space and outer membrane) contain proteinases that cleave casein components at different rates. Proteinases of the intermembrane space preferentially cleave κ -casein, whereas the proteinases of the outer membrane, inner membrane and matrix fractions degrade α_{s1} -casein more rapidly. Electrophoretic separation of the degradation products of α_{s1} -casein and κ -casein in polyacrylamide gels shows that different polypeptides are produced when the substrate is degraded by the matrix, by both membranes and by the intermembrane-space fraction. Some of the degradation products resulting from incubation of the caseins with the mitochondrial fractions are probably the result of digestion by contaminating lysosomal proteinase(s). The matrix has a high peptidase activity, since glucagon, a small peptide, is very rapidly degraded by this fraction. These observations strongly suggest that distinct proteinases, with different specificities, are associated respectively with the intermembrane space and with both membrane fractions.

INTRODUCTION

A great interest has developed in recent years in the mitochondrial proteinases and their role in mitochondrial processes. Excellent progress has been made in the field of the proteolytic processing of translocated mitochondrial precursors, some of the responsible proteinases having already been identified [for reviews, see Hay *et al.* (1984) and Schmidt & Neupert (1984)]. However, little attention has been paid to the degradative enzymes, associated with mitochondria, that may be responsible for the final elimination of proteins within the organelle.

We have previously reported the existence of proteinases active against casein in rat liver mitochondria (Duque-Magalhães & Régnier, 1982). In the present work we show that the membrane and the intermembrane-space fractions degrade the three main components of casein at different rates, leading to the formation of different peptides. These results demonstrate that the several mitochondrial casein-degrading enzymes of different specificities are not evenly distributed in the mitochondrial compartments. One of these enzymes, namely that associated with the intermembrane space, can be differentiated from other proteinases because of its particular specificity towards κ -casein. Some mitochondrial proteolytic enzymes appear to be of membrane origin; associated with either the inner or the outer membrane, these enzymes seem to have very close specificities.

MATERIALS AND METHODS

Chemicals

Total casein (Hammarsten, BDH), α_{s1} -casein, κ -casein (a gift from Dr. J. C. Gripon) and glucagon (Sigma), were iodinated with ^{125}I (The Radiochemical Centre, Amersham, Bucks., U.K.), using the chloramine-T method (Greenwood *et al.*, 1963).

Animals

Male Wistar rats, weighing 200–250 g, were used. The animals were fasted overnight and decapitated immediately before the experiment. The livers were perfused by injection with saline (0.9% NaCl) into the portal vein.

Preparation of mitochondria and of submitochondrial fractions

Mitochondria were prepared from rat livers by a modification of a conventional method that uses washing with low concentrations of digitonin in order to separate them from lysosomes (Duque-Magalhães & Braz, 1981). The purified mitochondria were fractionated into four submitochondrial fractions (matrix, inner membrane, intermembrane space and outer membrane) by a modification of the method of Schnaitman & Greenawalt (1968). The intermembrane-space fraction was collected after sedimentation of the outer membrane at 144 000 *g*. The mitoplasts, previously sedimented by centrifugation at 12 000 *g*, were sonicated with a MSE sonifier (3 × 30 s) and centrifuged at 144 000 *g* to separate the matrix from the inner membrane.

Preparation of lysosomal fractions

Lysosomes were isolated from the livers of rats injected with Triton WR 1339 as described by Trouet (1974).

Electrophoretic separation of the caseinolytic products

The incubation mixtures for degradation of casein substrates contained in 60 μl of phosphate buffer (10 mM, pH 7.5), 40 μg of ^{125}I -labelled total casein or 14 μg of ^{125}I -labelled pure casein (α_{s1} - or κ -casein) and an adequate amount of proteins from the subcellular fractions (50 μg for each of the submitochondrial fraction and 20 μg of lysosomal proteins). Incubations were carried out at 37 °C; 10 μl aliquots were withdrawn at 0, 2 and 4 h and immediately added to 23 μl of Tris/HCl buffer (0.12 M, pH 6.8) containing 3% (w/v) SDS, 8%

(v/v) mercaptoethanol and placed in a boiling-water bath for 5 min. The samples were then applied to SDS/20%-(w/v)-polyacrylamide slab gels (0.71 mm thick). Electrophoresis was performed by the method of Laemmli (1970) for 18 h at constant voltage (50 V). The ^{125}I -labelled regions were located in the gel after autoradiography with Kodak NS-ST X-ray film.

Determination of the acid-soluble hydrolysis products

The proteolytic or peptidase activities were determined by measuring the acid-soluble radioactivity liberated by hydrolysis of the ^{125}I -labelled substrates. The incubation mixtures contained, in 120 μl total volume, 6 μmol of phosphate buffer, pH 7.5, 28 μg of ^{125}I -labelled pure casein (α_{S1} -casein or κ -casein) or 50 μg of ^{125}I -labelled glucagon and 100 μg of proteins from a submitochondrial fraction. Incubations were carried out at 37 °C and stopped at different times by addition of 30 μl of 50% (w/v) trichloroacetic acid. After 30 min at 0 °C, the precipitate was removed by centrifugation and the acid-soluble radioactivity measured in a γ -radiation counter (Picker-Pace 1).

RESULTS AND DISCUSSION

Comparison of the different degradation rates for the casein components

The proteolytic activity of the four fractions resulting from the separation of the mitochondrial compartments were assayed against ^{125}I -labelled total casein and, after electrophoretic separation of the incubation mixtures on a polyacrylamide gel, the degradation rates for the casein components (α_{S1} -casein, β -casein and κ -casein) were deduced from the decreasing intensities of the spots corresponding to these polypeptides on autoradiograms (results not shown).

In general, α_{S1} -casein was the component more efficiently degraded by the mitochondrial proteinases, except for the proteinases in the intermembrane space, which preferentially cleaved the κ -casein.

Quantification of the degradation rates for pure casein components

A quantitative estimation of the degradation rates of α_{S1} -casein and κ -casein was achieved by measuring the increase in acid-soluble peptides that occurs when pure ^{125}I -labelled caseins (α_{S1} or κ) are incubated in the presence of the enzymic extracts (Table 1).

This experiment clearly established that the inter-

membrane-space fraction contains proteinase(s) that degrade κ -casein more efficiently than α_{S1} -casein, in contrast with all the other mitochondrial fractions, which contain proteinase(s) that cleave α_{S1} -casein more rapidly.

Identification of the products from degradation of α_{S1} -casein and κ -casein

Proteinases of different specificities can be differentiated because they cleave different peptide bonds in a polypeptide chain and this results in degradation products of different sizes. Consequently the gel pattern obtained after electrophoretic separation of the cleavage products from a well-defined substrate will be characteristic of a proteinase or mixture of proteinases.

Upon incubation with α_{S1} -casein or κ -casein, the four mitochondrial fractions give rise to several degradation products (bands A, B, C, D and E in Fig. 1 and bands K, L, M, N and O in Fig. 2). Some of the bands migrate in SDS/polyacrylamide gels in the same way as the polypeptides produced by incubating the radioactive substrates with lysosomal extracts (e.g. bands A and E in Fig. 1 and bands K and N in Fig. 2). This could reflect the presence, in both mitochondria and lysosomes, of proteinases of close specificities that cleave α_{S1} - and κ -casein at the same structural feature. In spite of a very careful purification this could also occur because of a residual contamination of mitochondrial preparations by lysosomal proteinases.

For this reason we only considered as characteristic of the degradation of α_{S1} - and κ -casein by mitochondrial proteinases the polypeptides that do not migrate in the gels at the same rate as the degradation products obtained by degrading the same substrate with the lysosomal fraction.

All the degradation products resulting from incubation of α_{S1} or κ -casein with the matrix fraction have the same localization in the gels as those resulting from degradation with lysosomal proteinases. Therefore we can not exclude the possibility that most of the proteolytic activity of the matrix fraction comes from lysosomal contamination.

The gel patterns are very similar when radioactive pure caseins are degraded by either the inner- or the outer-membrane fraction. Some of the products obtained, namely band A in Fig. 1 and band N in Fig. 2, could indicate cleavage by lysosomal enzymes, especially in the case of those from the inner membrane.

Nevertheless two degradation products, the band-D α_{S1} -casein peptide and the band-M κ -casein peptide are generated only by membrane fractions (Figs. 1 and 2). It

Table 1. Proteolytic activities of submitochondrial fractions towards α_{S1} -casein and κ -casein

Caseinolytic activities are expressed as μg of ^{125}I -labelled α_{S1} -casein or κ -casein hydrolysed/h per mg of enzymic protein. The incubation mixtures contain, in 120 μl , 28 μg of ^{125}I -casein, 100 μg of protein from each mitochondrial fraction and 6 μmol of phosphate buffer, pH 7.5. The radioactivity was counted after centrifugal separation of non-degraded casein.

Substrate	Mitochondrial fraction...	Activity ($\mu\text{g}/\text{h}$ per mg)			
		Matrix	Inner membrane	Intermembrane space	Outer membrane
α_{S1} -Casein		15	31	7	14
κ -Casein		9	26	16	11

Fig. 1. Degradation of α_{s1} -casein by the four mitochondrial fractions and a lysosomal fraction

Each of the mitochondrial fractions (50 μ g of protein) and the lysosomal fraction (19.5 μ g of protein) was incubated with 12 μ g of 125 I-labelled α_{s1} -casein in 60 μ l of phosphate buffer (50 mM, pH 7.5) for 2 and 4 h. The incubation mixtures were applied to SDS/20%-(w/v)-polyacrylamide slab gels. After electrophoresis the gels were autoradiographed. The autoradiogram in this Figure shows the bands (letters A–F) corresponding to the degradation products of α_{s1} -casein (α_{s1}) by the matrix (mat.) (lanes 2 and 3), inner-membrane (i.m.) (lanes 4 and 5), intermembrane-space (inter.) (lanes 6 and 7), outer-membrane (o.m.) (lanes 8 and 9) and a lysosomal fraction (Lys.) (lanes 10 and 11).

is noteworthy that no band has been observed that is specifically produced by only one of the membrane fractions. This indicates that mitochondrial casein-degrading proteinases are specifically associated with the membranes and have the same specificities whether bound to the inner or the outer membrane.

When incubated with α_{s1} -casein, the intermembrane-space fraction also produces polypeptides with migration rates identical with others obtained with either the membrane fraction (band C) or the lysosomal fraction (band A) as proteinase source. However, when κ -casein was used as substrate, the intermembrane-space fraction gave rise to a characteristic polypeptide (band L) that is not generated by any other mitochondrial or lysosomal fraction.

Fig. 2. Degradation of κ -casein by the four mitochondrial fractions and a lysosomal fraction

Each of the mitochondrial fractions and a lysosomal fraction are incubated with 125 I-labelled κ -casein under the conditions described in the legend of Fig. 1. The autoradiogram in this Figure shows the bands (letters K–O) corresponding to the degradation products of κ -casein (κ) by the matrix (lanes 2 and 3), inner-membrane (lanes 4 and 5), intermembrane-space (lanes 6 and 7), outer-membrane (lanes 8 and 9) and a lysosomal fraction (lanes 10 and 11). Other abbreviations are defined in the legend to Fig. 1.

This reveals the existence of a soluble mitochondrial proteinase specifically localized in the space between the inner and the outer membranes.

The degradation pattern in Fig. 2 shows that the outer-membrane, inner-membrane and the intermembrane-space fractions cleave κ -casein to polypeptides that accumulate in the gel. In contrast, very few polypeptides are present in the gel pattern corresponding to the degradation of κ -casein by the matrix fraction.

Measurements of peptidase activities using glucagon as substrate

We have measured the peptidase activity of the four mitochondrial fractions by using glucagon, a 29-amino-

Table 2. Degradation of glucagon by the mitochondrial fractions

The degradation of 125 I-glucagon was measured as described in Table 1.

Mitochondrial fraction	Specific activity (μ g of glucagon/h per mg of protein)	Total activity (μ g of glucagon/h per g of liver)
Matrix	265	3127
Inner membrane	175	1041
Intermembrane space	149	687
Outer membrane	67	133

acid-long peptide, as substrate. From the results in Table 2 it can be seen that the matrix fraction contains the highest peptidase activity (expressed either as total or as specific activity).

This is probably accounted for by the high content of peptidases in this mitochondrial fraction which, by rapidly degrading the primary products of casein cleavage, hinder any accumulation of intermediate polypeptides. The inner-membrane fraction also contains a higher peptidase activity than those exhibited by the outer-membrane or the intermembrane-space fractions.

From these results it can be concluded that rat liver mitochondria contain several proteinases, of different specificities, that are associated either with the intermembrane space or with both membrane fractions. Peptidase activity is mainly located in the matrix.

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