THE LOCALIZATION OF A PROTEINASE WITHIN RAT LIVER MITOCHONDRIA

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SUMMARY

A membrane fraction containing a proteinase was separated from rat liver mitochondria by centrifugation through 1.7 M sucrose. The submitochondrial localization of the proteinase was achieved by fractionation of mitochondria into outer membrane, inner membrane, and matrix, characterized by their marker enzymes monoamine oxidase, cytochrome c oxidase and malate dehydrogenase, respectively. The proteinase was found exclusively in the inner membrane fraction. Experiments, which exclude a lysosomal contamination are described.

INTRODUCTION

The presence of a proteinase in mitochondria has been postulated by several authors (1-6). Until now no such enzyme could unambiguously be shown in mitochondria. Katunuma and coworkers (7) purified a proteinase which acts on the apoproteins of pyridoxal phosphate dependent enzymes from rat liver starting the purification with a mitochondrial fraction. In the course of our studies on the degradation of chromosomal proteins we have found and purified a proteinase from a mitochondrial fraction (8,9). Its mitochondrial localization, however, was not clearly demonstrated.

In the following paper we report the presence of a protein-

ase within rat liver mitochondria and its localization in the inner membrane. The importance of its physiological function is discussed.

MATERIALS AND METHODS

Isolation of mitochondria and preparation of submitochondrial fractions.

Mitochondria were prepared from livers of male Wistar rats as described by Loewenstein et al. (10) with the following exception. A 0.07 M sucrose, 0.22 M mannitol and 2 mM hepes (N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid) buffer, pH 7.4 was used (11) instead of 0.25 M sucrose in 1 mM Tris/HCl, pH 7.5 buffer. The outer and intermembrane fractions were prepared by the digitonin procedure of Greenawalt (11). For the separation of the inner membrane and the matrix fraction, mitoplasts were sonicated (Branson Sonifier, micro-tip 3x10 sec) and centrifuged at 150 000 g_{av} for 1 h. The procedure for the preparation of the heavy mitochondrial membrane fraction has been previously described (9).

Enzyme assays.

Proteinase activity was measured with N-acetyltyrosine ethylester (ATEE) in a coupled assay with alcohol dehydrogenase (9). Monoamine oxidase was assayed spectrophotometrically at 250 nm with benzylamine as a substrate (12). The cytochrome c oxidase assay was carried out according to the procedures described by Smith (13). Malate dehydrogenase was determined as described by Wolfe and Neilands (14).

RESULTS

When mitochondria were layered on a discontinuous sucrose gradient and centrifuged, a heavy membrane fraction with proteinase activity was found in the pellet (9). In order to establish the submitochondrial localization of this proteinase, purified mitochondria have been separated into outer, inner, and inter membrane fractions, and the matrix. The fractions were characterized by their marker enzymes, monoamine oxidase, cytochrome c oxidase and malate dehydrogenase. It can be seen from table 1 that the proteinase is found in the

Table 1 Distribution of the proteinase in submitochondrial fractions

fraction	monoamine oxidase			malate dehydrogenase			cytochrome c oxidase			proteinase		
	sp.act.	total units	total units	sp.act.	total units	total units	sp.act.	total units	total units	sp.act.	total units	total units
	mU/mg	U	%	mU/mg	U	%	mU/mg	U	%	mU/mg	U	%
mitochondria	14	7.28	100	1510	732	100	265	122	100	0.0	-	-
outer membrane	34.5	1.16	16	768	36.3	5.0	183	8.9	7.3	0.0	-	-
intermembrane fraction	13.8	0.45	6.2	488	94.4	12.9	0.0	-	-	0.0	-	-
inner membrane	0.0	-	-	1015	136	18.6	380	182	149	49	5.2	100
matrix	0.0	-	-	2860	467	63.8	0.0	-	-	0.0	-	-
heavy membrane fraction	0.0	-	-	1038	4.3	0.6	77	0.250	0.2	397	1.5	29
heavy membrane fraction re-centrifuged	0.0	-	-	1.3	0.001	-	23.3	0.012	-	2308	1.1	21

The assays for monoamine oxidase, malate dehydrogenase, cytochrome c oxidase, and proteinase are given in Materials and Methods. The total activity of cytochrome c oxidase of the submitochondrial fractions exceeds the value obtained for mitochondria since cytochrome c oxidase is probably not totally accessible to its substrate. The data are average values of at least 4 experiments.

inner mitochondrial membrane fraction. In accordance with the localization of the proteinase in the inner mitochondrial membrane is the fact that intact mitochondria do not show proteolytic activity in the assay with N-acetyltyrosine ethyl-ester as substrate. Since we have purified the proteinase from a heavy mitochondrial membrane fraction (9), it was necessary to show that this insoluble enzyme is the same as that localized in the inner mitochondrial membrane. It is shown in table 1 that the heavy membrane fraction, obtained after discontinuous sucrose density centrifugation of mitochondria, exhibits an 8-fold increase in the specific activity of the proteinase compared to the enzyme from the inner mitochondrial membrane. The presence of cytochrome c oxidase in the heavy membrane fraction is regarded as strong evidence for the localization of the proteinase in the inner mitochondrial membrane. After recentrifugation of the heavy membrane fraction through 1.7 M sucrose the specific activity of the proteinase was increased 6-fold, whereas practically all of the cytochrome c oxidase activity is removed (table 1).

Although there was no detectable acid phosphatase activity in the mitochondrial fraction, the following control experiments have been carried out in order to exclude the possibility of contamination by soluble or membrane bound lysosomal proteinases. Therefore, lysosomal membranes were prepared from tritosomes (15), which were either frozen and thawed, or sonicated, or treated with 0.6 % digitonin, conditions which were used for the disintegration of mitochondria. None of the differently treated lysosomal membranes showed proteolytic activity in the assay with N-acetyltyrosine ethyl-

ester as substrate. We have shown that the soluble lysosomal proteinases have a pH-optimum of around 3-4 in a histone degrading assay system (8). Inspite of this low pH optimum there is still measurable proteinase activity at pH 8. This proteinase activity could be due to a proteinase with a pH optimum of 8, which may be a low activity component of the lysosomal proteinases. To exclude the possible contamination of mitochondria by this proteolytic activity the following experiments were done. Freshly prepared lysosomes were mixed with liver slices before the preparation of mitochondria and subsequent sucrose density centrifugation. No difference in the specific activity and yield of the heavy mitochondrial membrane fraction was found.

It is known from our previous studies that soybean trypsin inhibitor is a powerful inhibitor of the proteinase activity (8). If a contaminating lysosomal proteinase was attached to the mitochondrial surface the proteolytic activity should be inhibited upon addition of soybean trypsin inhibitor. No change in specific activity and yield of the proteinase from the heavy mitochondrial membrane fraction could be demonstrated after incubation of mitochondria with soybean trypsin inhibitor. A lysosomal contamination seems therefore unlikely.

DISCUSSION

The mitochondrial proteinase described in the present paper was discovered during our studies on the degradation of chromosomal proteins (8). We have presented biochemical evidence that the enzyme is localized within the inner mitochondrial membrane. Most of the mammalian intracellular pro-

teinases described so far are soluble and relatively unspecific enzymes. The proteinase of the present study, however, is found in the inner mitochondrial membrane, and shows a high degree of substrate specificity (9). Whether the enzyme plays a regulatory role in the assembly of inner membrane components during the biogenesis of mitochondria or in the turnover of the inner mitochondrial membrane remains to be demonstrated.

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