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CYTOCHROME *C* DEGRADING ACTIVITY IN RAT LIVER MITOCHONDRIA

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SUMMARY

By means of a suitable zymographic technique we revealed a cytochrome *c* degrading system in rat liver mitochondria. After separation of the mitochondrial proteins by polyacrylamide gel electrophoresis and incubation of the gels in the presence of cytochrome *c* , three bands of protease activity were detected. Distinct zymogram patterns were obtained in parallel assays using lysosomes or cytosol instead of mitochondria. The soluble and the membrane components of mitochondria were analyzed by the same method to assess the intra-mitochondrial localization of these proteases.

The possible occurrence of a neutral protease activity in rat liver mitochondria was originally suggested by ALBERTY et al. (1) on the basis of a marked autoproteolysis exhibited by these fractions at neutral pH. More recently, data suggesting the existence of a proteolytic system in mitochondria have been reported (2-4), although the subject remains controversial, since arguments have also been raised against the true mitochondrial nature of such proteases (5-7).

Further elucidation of this problem is of importance as a proof of the existence of proteolytic enzymes tied to particular structures could bring a new outlook on the mechanism of the degradation of intracellular proteins.

In the present paper we report experimental evidence of the mitochondrial localization of a proteolytic system active towards cytochrome *c* in a neutral pH range. This finding might be significant inasmuch as it was recently shown that some mitochondrial cytochromes have larger precursors in the cytoplasm which must

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be processed to smaller peptides before being imported into mitochondria, likely involving a proteolytic step (8).

METHODS

Animals : Two months old Wistar rats were fasted overnight and decapitated immediately before the experiment. Livers were perfused with ice cold saline (9).

Preparation of subcellular fractions : Immediately after the perfusion livers were homogenized in 9 vol. of 0.25 M sucrose in 1 mM Tris-HCl pH 7.0. Mitochondria were prepared as described by LOEWENSTEIN (10). For the separation of the mitochondrial membranes from the soluble part we used hypotonic lysis of mitochondria in water followed by 105,000xg centrifugation as described by CHAPMAN (11). Lysosomes were isolated 3.5 days after injection of Triton RW 1339 into 200g rats (12). Cytosol was obtained by centrifuging the post-mitochondrial fraction at 105,000xg, 1 hr.

Acid Phosphatase Assay : 50 µl of the enzyme sample was preincubated 10 min at 37°C with 1.45 ml of 0.2 M sodium acetate/ /acetic acid buffer, pH 5.0 ; 0.5 ml of 0.32 M p-nitrophenylphosphate substrate was added and after 60 min incubation the reaction was stopped with 2 ml ice cold 1 M Tris/HCl pH 8.5, containing 0.4 M K_2HPO_4. Samples were read at E_{420} immediately.

Protein determination : Proteins were estimated by the Lowry method (13), using LAB-TROL (from Dade Co., Miami, FL) as a standard.

Preparation of the samples for electrophoresis : The subcellular fractions were resuspended in Tris-HCl buffer (pH 7.25) 0.05 M and Triton X-100 added to a final concentration of 0.5%. After solubilization (30 min at 0°C) the solutions were made dense with 30% sucrose and a sample of 100 µg of protein was applied per gel.

Acrylamide gel electrophoresis : Polyacrylamide gels (7.0% acrylamide) without spacer, were prepared according to the method of Davis (14). A constant current of 3 mA/tube was applied during 90 min. Control gels were stained for protein with Coomassie Brilliant Blue (15).

Detection of proteolytic activities in polyacrylamide gels : For direct detection of proteolytic activity, cytochrome *c* was used as substrate. After electrophoresis the gel was removed, washed twice in Tris-HCl buffer (pH 7.25) 0.05 M, for 30 min, at 0°C and placed in a vial (10 mm x 150 mm) and 5.0 ml of substrate solution was added to completely immerse the gel. The substrate solution comprised 1.0 ml of 8% cytochrome *c* in 8 M urea and 3.0ml of 50 mM Tris-HCl pH 7.25 containing 1.7 mM $CaCl_2$ (16). The vial was closed and the gel immersed in this protein solution for 1 hr at 37°C. The substrate was then withdrawn, the tube closed and the gel incubated for a further 1 hr at 37°C. The gel was then placed in 12.5% TCA to fix the undigested absorbed protein. Proteinase bands appear as transparent zones against an evenly precipitated brown background, as described by the method in (17).

RESULTS

The mitochondrial fractions utilised in our experiments were treated with digitonin and have a minimal lysosomal contamination as evaluated by striking reduction of the marker enzyme acid phosphatase. In fact less than 14% of the initial acid phosphatase activity remained in the final mitochondria preparation after the digitonin treatment.

Electrophoretic separation of the mitochondrial proteins in polyacrylamide gels, with subsequent staining by Coomassie Brilliant Blue revealed a complex pattern containing at least 24 components (not shown). Among these bands, only three showed proteolytic activity as visualised by clear digestion bands in gels impregnated with cytochrome *c* after electrophoresis and incubated for proteinase digestion (Fig. 1). Fig. 1 also presents parallel assays with the soluble and membrane sub-fractions of the mitochondria, performed in order to assess the

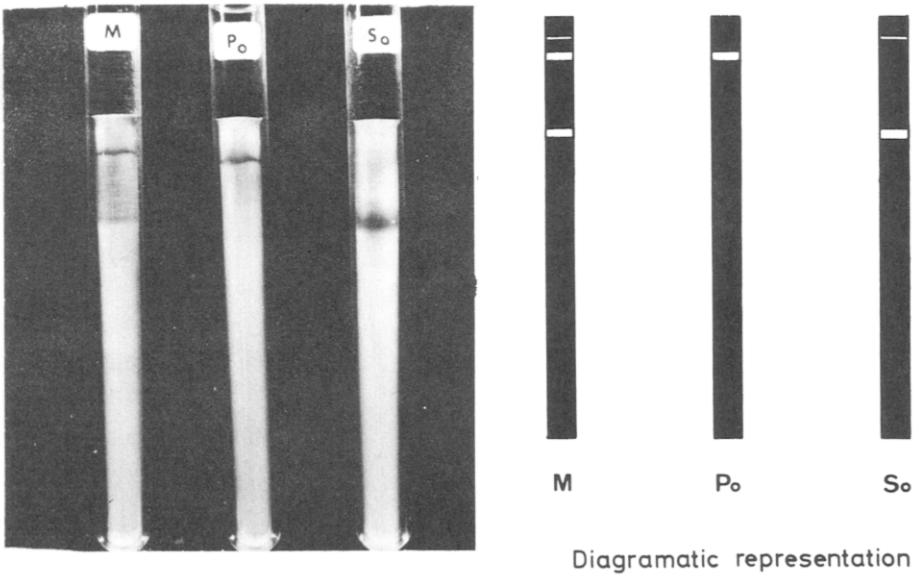

Fig. 1 Zymogram patterns of Triton X-100 solubilized mitochondria (M), membrane (P_o) and soluble (S_o) fractions.

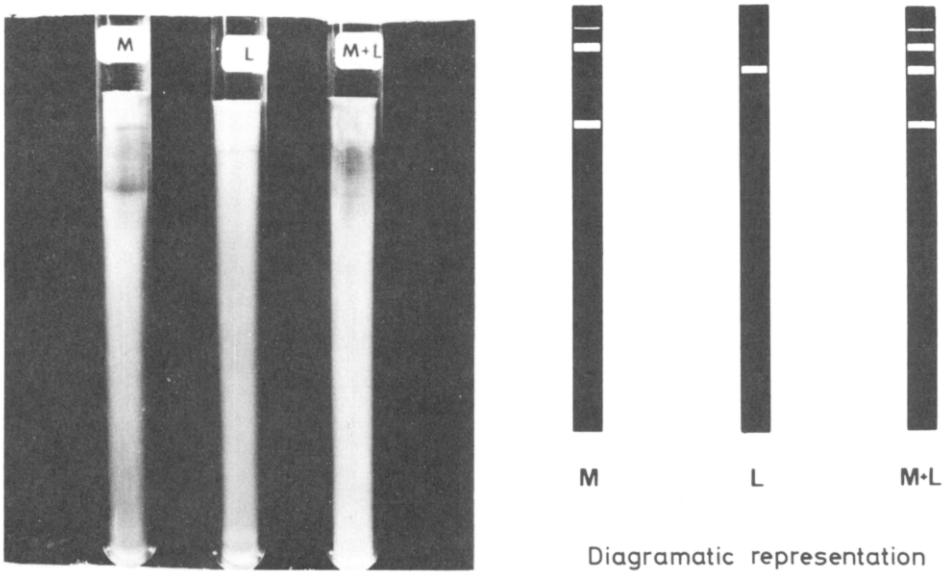

Fig. 2 Zymogram patterns of Triton X-100 solubilized mitochondria (M), lysosomes (L) and pool of both fractions (M+L).

intra-mitochondrial distribution of these proteases. Two digestion bands against cytochrome *c* invariably appeared in the soluble fraction, while the membrane fraction always showed a unique band. The sum of these two patterns corresponds to the original mitochondria zymogram.

Fig. 2 compares the proteolytic activity towards cytochrome *c* , of the mitochondrial (M) and lysosomal (L) fractions. Zymogram (L) exhibits only one digestion band which is quite distinct from the mitochondrial one as depicted in a third zymogram (M+L) corresponding to a pool of these two fractions.

Fig. 3 shows in a similar way the differences between mitochondrial (M) and cytosol (C) proteases and also depicts a zymogram of the pooled fractions. Multiple cytochrome *c* degrading enzymes exist in the cytoplasm, but their concentrations are certainly much inferior than in mitochondria, since for the same protein load on the gel the cytoplasmic proteases originate dif-

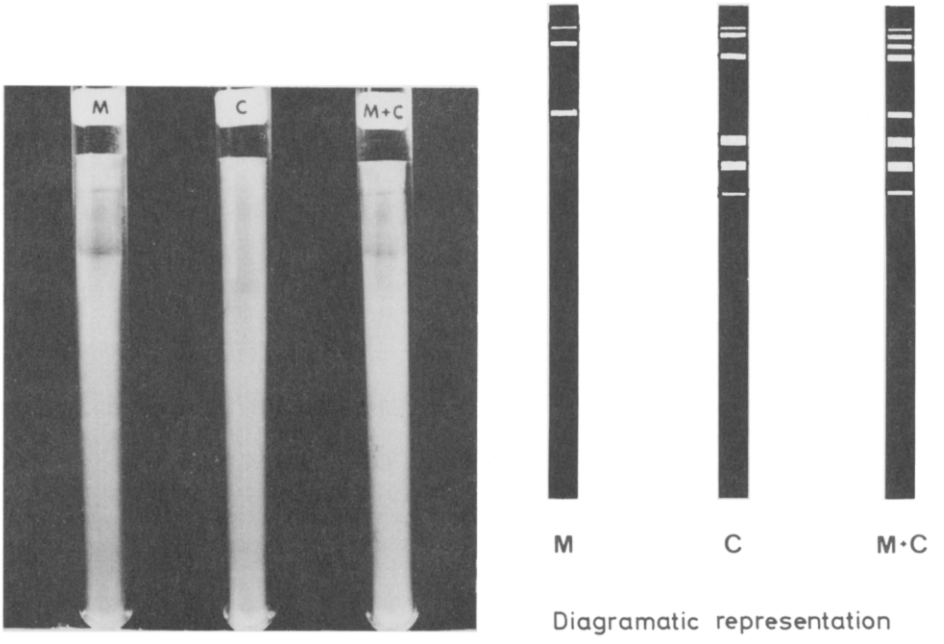

Fig. 3 Zymogram patterns of Triton X-100 solubilized mitochondria (M), cytosol (C) and pool of both fractions (M+C).

fused digestion bands which do not allow clear photographic reproduction. However these regions of proteolytic activity are detectable in positions of the zymogram which are consistently reproducible and different from those detected with mitochondria.

DISCUSSION

Our study was based on the direct detection of the proteolytic activity after separation of the mitochondrial proteins by electrophoresis in polyacrylamide gels. Cytochrome *c* was chosen as a suitable substrate not only because of its presumable specificity for the system examined, but also because it requires no staining. By this method we were able to demonstrate in rat liver mitochondria a proteolytic system active in the neutral range of pH towards cytochrome *c*. Three digestion bands were invariably shown in a sample of digitonin treated

mitochondria. The intra-mitochondrial localization of these three proteases was approached by testing in parallel the membrane and soluble sub-fraction. One of the proteases of the mitochondrial preparation is revealed as membrane bound while the other two are apparently soluble enzymes.

Lysosomal extracts show a distinct proteolytic system towards cytochrome *c* . In fact none of the mitochondrial proteases revealed against this substrate seems to exist in lysosomes. The simplicity of the lysosomal zymogram consisting of only one digestion band is likely to be related to the mitochondrial nature of the substrate used as well as to the neutral pH of the assay. The lysosomal contamination of the mitochondria never exceeds 14%, which in the assay conditions represents a lysosomal protein load not greater than 10 µg per each mitochondrial gel. Thus it is not unexpected that it will not reproduce the characteristic lysosomal band in the mitochondrial gel.

The zymogram patterns of cytochrome *c* degrading enzymes are distinct in mitochondria and cytosol. Moreover the concentration of these proteases in cytoplasm has been shown to be much lower than in mitochondria. This can be taken as a further argument for a true mitochondrial nature of the proteolytic system we are describing, as it becomes difficult to conceive these proteases as mere contaminations if they are not dispersed in the cytoplasm.

The proteases reported in this paper may play a role in several regulatory mechanisms of the mitochondria, namely in connection with the processing of proteins to be imported into this organelle.

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REFERENCES

1. Alberty, K.G.M.M. and Bartley, W. (1969) Biochem. J. 111, 763-776
2. Hare, J.F. (1978) Biochem. Biophys. Res. Commun., 83, 1206-1215
3. Haas, R. and Heinrich, P.C. (1978) Eur. J. Biochem., 91, 171-178
4. Duque-Magalhães, M.C. (1979) FEBS Lett., 105, 317-321
5. Ferdinand, W., Bartley, W. and Broomhead, V. (1973) Biochem. J., 134, 431-436
6. Rubio, V. and Grisolia, S. (1977) FEBS Lett., 75, 281--284
7. Woodbury, R.G., Gruzensky, G.M. and Lagunoff, D. (1978) Proc. Natl. Acad. Sci. USA, 75, 2785-2789
8. Côté, C., Solioz, M. and Shatz, G. (1979), J. Biol. Chem., 254, 1437-1439
9. Ross, B.D. (1972) Perfusion techniques in Biochemistry, p. 184, Clarendon Press, Oxford
10. Loewenstein, J., Scholte, H.R. and Wit-Peeters, E.M. (1970) Biochim. Biophys. Acta, 223, 432-436
11. Chapman, M.J., Miller, L.R. and Ontko, J.A. (1973) J. Cell Biol., 58, 284-306
12. Trouet, A. (1974) Meth. Enzymol. 31A, 323
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem., 193, 265-275
14. Davis, B.J. (1964) Ann. N.Y. Acad. Sci., 121, 404
15. Higerd, T.B. and Spizizen, J. (1973) J. Bacteriol., 114, 1184-1192
16. Laskowski, M. (1955) Meth. Enzymol., 2, 26-36
17. Ward, C.W. (1976) Anal. Biochem., 74, 242-245