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PARTICIPATION OF THE INNER MITOCHONDRIAL MEMBRANE IN THE INACTIVATION OF CARBAMYL PHOSPHATE SYNTHASE AND OF ADENOSINE TRIPHOSPHATASE BY LYSOSOMES

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SUMMARY: Rat liver carbamyl phosphate synthase (EC.2.7.2.5) and adenosine triphosphatase (EC.3.6.1.3) are inactivated at or near intracellular pH by component(s) of the inner mitochondrial membrane in combination with lysosomal enzymes. At the pH range 6-7.4, lysosomal enzymes have little or no effect on carbamyl phosphate synthase and particularly on adenosine triphosphatase. However, at this pH adenosine triphosphatase, which has been exposed first to inner membrane preparations, is inactivated more rapidly on addition of lysosomal preparations. The inactivation is time and inner membrane concentration dependent. The inactivation by inner membrane appears to result in limited or minimal proteolysis as judged by release of acid soluble radioactivity using labelled carbamyl phosphate synthase. Thus, it appears that the inner membrane plays an initial role in the degradation of some mitochondrial proteins.

The mechanisms responsible for the turnover of intracellular proteins are not known. It has been suggested that both lysosomal and non-lysosomal pathways of protein degradation are operative (1,2) and several schemes have been proposed (3-8). It has been shown that even at neutral pH lysosomal components could be responsible for the bulk of mitochondrial protein degradation. However, it has also been shown that mitoplasts possess a residual proteolytic activity which could also be responsible for protein degradation (4). This dilema has now been clarified; although lysosomes are potentially able to degrade most proteins at low pH, as shown in this paper the inner mitochondrial membrane participates in the inactivation and possibly initial degradation of two mitochondrial enzymes, i.e. carbamyl phosphate synthase and adenosine triphosphatase (ATPase), in the range of intracellular pH at which lysosomes per se are largely inactive.

Our choice of these enzymes was influenced by the facts that they are both mitochondrial enzymes but are located in different compartments, and that they

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have different half-lives (4) but similar molecular weights. As shown here, both enzymes are inactivated by incubation with inner mitochondrial membrane which potentiates degradation by lysosomes. There is hardly any proteolytic degradation associated with the initial inactivation; however, on protracted incubation some proteolysis, i.e. production of acid soluble products, can be measured. It seems, therefore, that these findings can be interpreted as suggesting an initial role of the inner mitochondrial membrane in the degradation of some proteins.

MATERIALS AND METHODS. Male Wistar rats weighing 200-250 g were used. All chemicals were of analytical grade.

Rat liver mitochondria, mitoplasts and the digitonin-soluble fraction (containing the outer membrane and the inter-membrane space plus contaminating organelles, including lysosomes) were prepared according to Greenewalt (9). The mitoplasts were disrupted by suspension in 10 mM potassium phosphate buffer, pH 7.4, 5 mM 2-mercaptoethanol, followed by freezing and thawing 5 times. Inner membranes and soluble matrix proteins were separated by 30 min centrifugation at 40,000 x g. The inner membranes were washed twice with 10 mM potassium phosphate buffer, pH 7.4, 5 mM 2-mercaptoethanol and suspended in the same buffer. When indicated, membranes were further washed with ammonium sulfate.

Table I. Effect of lysosomes and of mitochondrial fractions from rat liver on carbamyl phosphate synthase activity.

Additions	% of carbamyl phosphate synthase activity left at 1 h	2 h
None	95	95
Lysosomes	90	90
Mitoplasts	80	73
Matrix	100	95
Inner Membrane	20	5
Soluble Intermembrane Proteins	40	30
Outer Membrane	90	90

All incubations were carried out as described in Materials and Methods. They contained 2.75 mg/ml carbamyl phosphate synthase and 18 mg protein/ml of the indicated mitochondrial fractions. Lysosomes and bovine serum albumin were added as necessary so that all incubation mixtures contained 0.05 units of N-acetyl-β-D-glucosaminidase activity and equal amounts of protein.

Lysosomes were prepared by the method of Ragab et al. (10) and disrupted as described for mitoplasts. The whole "disrupted" preparation was used as such; for compactness, when we speak of addition of lysosomes, we mean this disrupted preparation.

The biuret-deoxycholate technique (11) was used to measure protein in the mitochondrial fractions. For purified enzymes the Lowry method (12) was employed. Bovine serum albumin (fraction V. Sigma Chemical Co.) was used as a standard. N-acetyl-β-D-glucosaminidase was assayed as described by Findlay et al. (13).

Carbamyl phosphate synthase and ATPase were prepared from rat liver mitochondria according to the methods of Nicoletti et al. (14) and Catterall and Pedersen (15), respectively. The ATPase was stored in 50% ammonium sulfate, the bulk of which was removed by centrifuging the enzyme before assay.

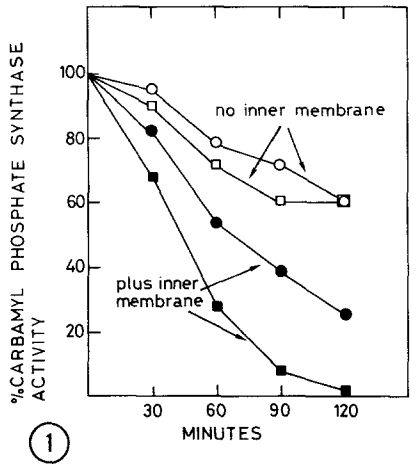
The incubations with carbamyl phosphate synthase were at 37°C and reaction mixtures contained in 10 mM potassium phosphate buffer, pH 7.4, 5 mM 2-mercaptoethanol, the enzyme and the mitochondrial fraction. Disrupted lysosomes and bovine serum albumin were added when necessary so that all incubation mixtures contained equal amounts of lysosomal proteolytic activity and total protein. At 0 time and at the indicated times, a portion was taken, diluted 1:10 in cold incubation buffer, centrifuged for 10 min at 12,000 x g and the supernatant assayed (16).

ATPase was incubated at 30 C with 170 mM potassium phosphate buffer, pH 6.0, 5 mM 2-mercaptoethanol, 2 mM EDTA and the mitochondrial fraction tested. After 40 min, an aliquot was added to an equal volume of a mixture containing 170 mM potassium phosphate buffer, pH 6.0, 5 mM 2-mercaptoethanol, 2 mM EDTA and lysosomes equivalent to 1.5 units of N-acetyl-β-D-glucosaminidase (1 unit of p-nitrophenol produced/ml/min) and further incubated. Aliquots of the incubation mixture containing lysosomes were taken at the indicated times and diluted 1:10 with 10 mM potassium phosphate, pH 7.4, 5 mM 2-mercaptoethanol at room-temperature. ATPase activity was assayed spectrophotometrically (15).

RESULTS AND DISCUSSION

Although lysosomes are potentially able, particularly at acid pH, to degrade almost any protein, it seems possible that some protein degradation may be carried out by non-lysosomal components and/or at pH's close to the intracellular pH (4). Also, since carbamyl phosphate synthase is not very stable at pH below neutrality, the studies reported here have been carried out at pH 7.4. ATPase is more stable, but because it becomes unstable below pH 6, most of our studies on stability with this enzyme have been carried out at pH 6.

Table I shows that carbamyl phosphate synthase is reasonably stable at pH 7.4 even to lysosomes, except in the presence of inner mitochondrial membrane (containing some lysosomal contaminant). It is of course very difficult to prepare pure cell fractions. Indeed, the soluble intermembrane fraction also yields appreciable inactivation of the enzyme probably due to solubilization and/or partial breakdown of the inner membrane during the digitonin treatment. The inner membrane present in the mitoplast fraction is likely responsible for the inactiva-



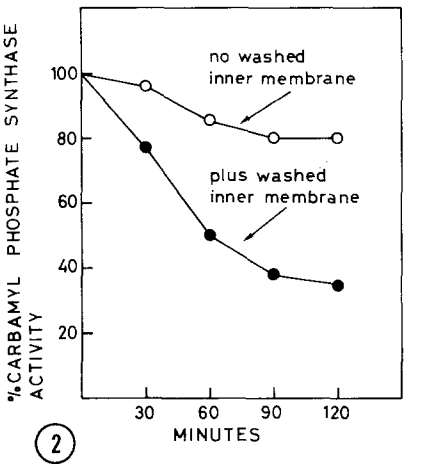


Fig. 1. Inactivation of carbamyl phosphate synthase by inner mitochondrial membranes. Incubations were at 37°C (see Materials and Methods) using 2.75 mg of carbamyl phosphate synthase in 1 ml. The samples were mixed with a lysosomal preparation containing 0.008 units (O-O) and 0.07 units (□-□) of N-acetyl-β-D-glucosaminidase or with 1.2 mg inner membrane protein (●-●) and 9 mg inner membrane protein (■-■).

Fig. 2. Inactivation of carbamyl phosphate synthase by inner mitochondrial membranes washed with 10% ammonium sulfate. Incubations were at 37°C (see Materials and Methods) and contained 2.75 mg of carbamyl phosphate synthase in 1 ml. The lysosomal activity and protein were in both cases, 0.05 units of N-acetyl-β-D-glucosaminidase and 14 mg, respectively. (O-O) no inner membrane protein. (●-●) 11.3 mg inner membrane protein.

tion shown. As illustrated, matrix and outer membrane seem largely inactive.

Fig. 1 shows that the inactivation of carbamyl phosphate synthase by inner mitochondrial membrane increases with time of incubation or with the amount of membrane added. Since, as already mentioned, the internal membrane preparation contains some lysosomal contaminant, we tried to minimize it by a number of procedures. Inner mitochondrial membrane washed with 10% ammonium sulfate loses approximately 75% of the lysosomal marker N-acetyl-β-D-glucosaminidase activity but retains the capability to inactivate carbamyl phosphate synthase. This is illustrated in Fig. 2.

Experiments with carbamyl phosphate synthase isolated from rats injected with (^{3}H)-L-leucine show that the inactivation by internal membrane is accompanied by a small release (ca. 3% in 24 h of incubation) of trichloroacetic acid soluble material, pointing to a limited proteolysis.

We next tested ATPase, because it has a molecular weight similar to that

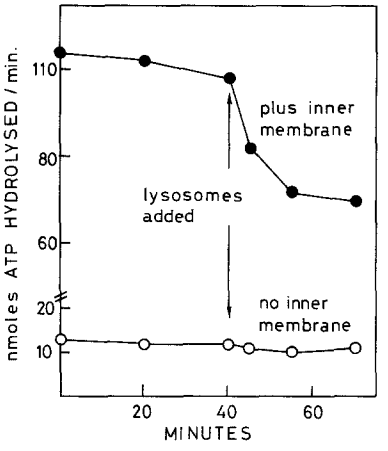


Fig. 3. Effect of lysosomes and of inner mitochondrial membrane from rat liver on ATPase activity. Purified ATPase (2.3 mg/ml) was incubated at 30°C with and without inner membranes for 40 min, then lysosomes were added as described in Materials and Methods and incubation continued as shown. When added, 15 mg membrane protein per ml were used.

of carbamyl phosphate synthase and because it is located in the membrane rather than in the mitochondrial matrix, as is carbamyl phosphate synthase. When ATPase was incubated in the presence of mitochondrial inner membrane there was some inactivation which was markedly increased after addition of disrupted lysosomes. It should be noted that the enzyme was stable to lysosomes in the absence of internal membrane, as shown in Fig. 3, and that it shows a lower sensitivity to lysosomes than carbamyl phosphate synthase. In other words, we had to use ca. 100 times more disrupted lysosomes to inactivate ATPase than carbamyl phosphate synthase, even in the presence of inner membrane. At pH 7.0 and above it remains active, even in the presence of massive quantities of lysosomes. This is of much interest also because the $t_{1/2}$ of carbamyl phosphate synthase is ca. 7-8 days while that of ATPase is ca. 2-3 days (4). Thus, factors other than pH and susceptibility to lysosomes are responsible for this difference in half-life.

In Table II, it can be seen that inner mitochondrial membranes washed with 10% ammonium sulfate retain the capability for inactivating ATPase. The Table also shows that the matrix fraction has little effect on the stability of ATPase, as is the case for carbamyl phosphate synthase.

Table II. Effect of lysosomes and of mitochondrial fractions from rat liver on ATPase activity.

Additions	% of ATPase activity left at 0 min	5 min	15 min
None	100	94	91
Matrix	100	92	85
Inner Membrane	90	79	67
Inner Membrane $(NH_4)_2SO_4$ washed	92	79	68

All incubations were carried out as described in Methods. They contained 2 mg/ml ATPase and when indicated 18 mg matrix protein/ml, 12.4 mg inner membrane protein/ml or 18 mg ammonium sulfate washed inner membrane. After 40 min incubation disrupted lysosomes containing 23 units of N-acetyl-β-D-glucosaminidase were added. Samples were taken and assayed at 0, 5 and 15 min. Incubation of the ATPase with the indicated amount of lysosomes, but without the inner membrane, did not inactivate the enzyme.

Since the inactivating effect shown by membranes is very heat labile and not easily extractable from the membrane (to be shown elsewhere), these results seem to indicate the presence of an enzyme(s) in the inner mitochondrial membrane which facilitates the inactivation of carbamyl phosphate synthase and of ATPase by lysosomes. Whether the inactivating enzyme is a protease and whether it has a role in the degradation of other mitochondrial proteins remains to be investigated.

Finally, it is of interest that the inactivation of carbamyl phosphate synthase is stimulated by ATP and GTP (to be published elsewhere), particularly since Goldberg and St. John (8) described an ATP-dependent proteolytic activity associated with cell membrane in E. coli.

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REFERENCES

1. Ballard, F.J. (1977) Essays Biochem. 13, 1-37.
2. Knowles, S.E. and Ballard, F.J. (1976) Biochem. J. 156, 609-917.
3. Grisolía, S., Knecht, E., Cervera, J. and Hernández-Yago, J. (1979) in Processing and turnover of proteins and organelles in the cell (Rapoport, S. and Schewe, F., eds) vol 53, pp 143-153, Pergamon Press, Oxford.
4. Grisolía, S., Knecht, E., Hernández-Yago, J. and Wallace, R. (1980) Ciba Foundation Symposium 75, 167-188, Elsevier/North Holland, Inc., New York.
5. Segal, H.L. (1976) in Lysosomes in Biology and Pathology (Dingle, J.T. and Dean, R.T., eds) vol 4, pp 295-302, North-Holland Publishing, Amsterdam.
6. Etlinger, J.D. and Goldberg, A.L. (1977) Proc. Natl. Acad. Sci. USA 74, 54-58.
7. Ciehanover, A., Hod, Y. and Hershko, A. (1978) Biochem. Biophys. Res. Comm. 81, 1100-1105.
8. Goldberg, A.L. and St. John, A.C. (1976) Ann. Rev. Biochem. 45, 747-803.
9. Greenewalt, J.W. (1976) in Methods in Enzymology (Fleischer, S. and Packer, L., eds) vol XXXI, pp 310-323, Academic Press, New York.
10. Ragab, H., Beck, C., Dilliard, C. and Tappel, A.L. (1967) Biochem. Biophys. Acta 148, 501-505.
11. Jacobs, E.E., Jacob, M., Sanadi, D.R. and Bradley, L.B. (1956) J. Biol. Chem. 223, 147-156.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
13. Findlay, J., Levy, G.A. and Marsh, C.A. (1958) Biochem. J. 69, 467-476.
14. Nicoletti, M., Guerri, C. and Grisolía, S. (1977) Eur. J. Biochem. 75, 583-592.
15. Catterall, W.A. and Pedersen, P.L. (1971) J. Biol. Chem. 240, 4987-4991.
16. Chabas, A., Grisolía, S. and Silverstein, R. (1972) Eur. J. Biochem. 29, 333-342.