LOCALISATION OF PROLINE OXIDASE AND Δ-PYRROLINE-5-CARBOXYLIC ACID DEHYDROGENASE IN RAT LIVER

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1. Introduction

The enzymes of proline-glutamate oxidation and the preparation of their substrates have been described by Strecker and coworkers [1, 3]. The presence of proline oxidase in rat liver mitochondria was described by Johnson and Strecker [2]. A rough localisation of proline oxidase and Δ-pyrroline-5-carboxylic acid dehydrogenase in locust flight muscle has been attempted by Brosemer and Veerabhadrappa [4]. Lang and Lang [10] reported proline oxidase to be bound to insoluble particles in rat liver cells.

This communication describes the localisation of both enzymes in rat liver by the methods of fractionated extraction [5, 6] and an improved method of preparing inner and outer membranes of rat liver mitochondria.

2. Materials and methods

2.1. *Fractionated extraction*

Rat liver tissue was fractionated by the method of Delbrück et al. [5] and Pette [6] which yields primarily a separation of extra- and intramitochondrial enzymes.

Abbreviations used:
PCDH = Δ-pyrroline-5-carboxylic acid dehydrogenase
MDH = malate dehydrogenase
SDH = succinic dehydrogenase
GLUDH = glutamate dehydrogenase
MAO = monoamine oxidase
ADK = adenylate kinase
TRA = triethanolamine hydrochloride

2.2. *Preparation of highly purified inner and outer mitochondrial membranes*

Rat liver mitochondria were prepared and treated as previously described by Neupert et al. [7], with the following modifications. After the step of swelling and shrinking, the mitochondrial suspension was sonified in small portions of 20 ml for 2 × 3 seconds with a Branson sonifier step 3. This gentle sonication is supposed to separate the loosely attached outer membranes from the inner membrane after the swelling-shrinking procedure. The suspension (5 ml) was then put on a 10 step discontinuous sucrose gradient of total volume 50 ml, increasing from 30% to 55% by 2.5% at each step. After centrifugation using the Beckmann SW.25.2 at 1° for one hour at 25 000 rpm bands were formed at each step which were collected and estimated for enzyme content.

Assay of enzymes: Monoamine oxidase, succinic dehydrogenase, adenylate kinase, glutamate dehydrogenase and malate dehydrogenase were estimated as described by Brdiczka and coworkers [8]. Proline oxidase and Δ-pyrroline-5-carboxylic acid dehydrogenase were estimated according to Brosemer and Veerabhadrappa [4]. Protein was measured by the biuret method of Beisenherz et al. [11]. Mitochondrial compartmentation was achieved by the methods of Schnaitman [9] modified by Brdiczka and coworkers [8].

L-proline was obtained from Calbiochem. Δ-pyrroline-5-carboxylic acid hydrochloride was synthesised according to Strecker [3].

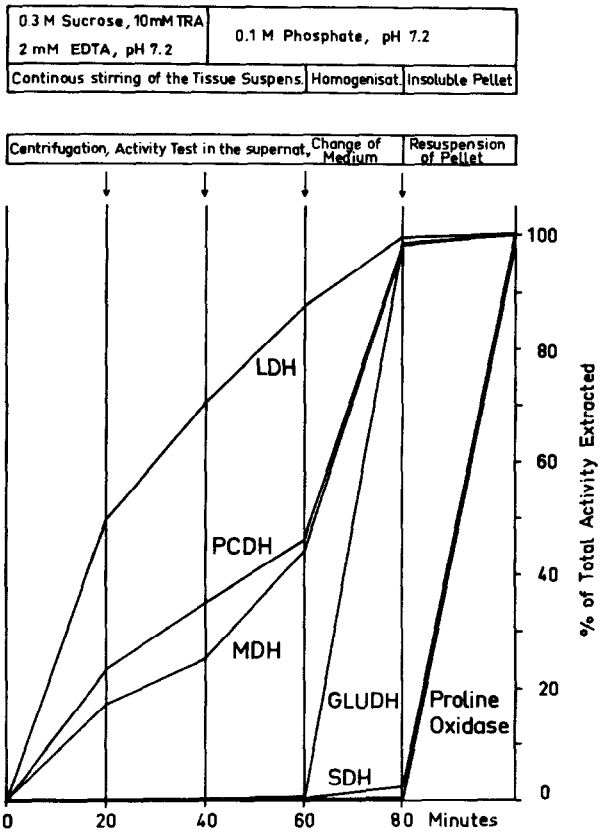

Fig. 1. Fractionated extraction of extra- and intramitochondrial enzymes of rat liver. The percentage of the total cellular activity extracted at different steps is plotted against the time course of the extraction.

3. Results

In fig. 1 is shown the behaviour of proline oxidase and Δ-pyrroline-5-carboxylic acid dehydrogenase during fractionated extraction. Repeated extractions with increasing osmotic and mechanical aggressiveness disclose successively different cellular compartments [6]. Enzymes of well defined location MDH, GLUDH, SDH and MAO have been measured for control. As is clearly seen proline oxidase is particle bound and is not even released after sonication in phosphate buffer. By separate cell fractionation we could confirm the findings of Johnson and Strecker [2] that in the liver cell proline oxidase does only occur in mitochondria. Thus proline oxidase must be bound

Table 1
Mitochondrial proteins after 3 min sonication (Branson sonifier step 5) and centrifugation at 144 000 × *g* for 60 min.

	Proline oxidase	Δ-pyrroline-5-carboxylic acid dehydrogenase
Supernatant	2%	95%
Insoluble fraction	98%	5%

to mitochondrial membranes. This is also shown in table 1 where soluble and insoluble mitochondrial proteins were separated and their enzyme content estimated.

Δ-pyrroline-5-carboxylic acid dehydrogenase behaves quite differently as shown in table 1 and fig. 1. It is not only a soluble enzyme which shows no attachment to particles but as is seen in fig. 1 it seems to be not entirely located in mitochondria but also to a certain extent in the cytoplasm. It runs almost parallel to malate dehydrogenase from which is known that there exist a cytoplasmic as well as a mitochondrial enzyme. A separation of cytoplasm was achieved by homogenising 1 part of liver with 7 parts 0.33 M sucrose medium in a Thomas tissue grinder 4288-B-C. The teflon pestle with a clearence of 0.006–0.009 inches was moved up and down 4 times spinning at 300 rpm. The homogenate was then centrifuged at 12000 × *g* for 10 min and revealed that about 50% of Δ-pyrroline-5-carboxylic acid dehydrogenase are found in the cytosole (table 2).

Whether it is the same enzyme or whether the enzyme found in mitochondria differs from that in the cytosole needs to be investigated. The question to which mitochondrial particle proline oxidase is bound was answered by a 10 step discontinuous sucrose gradient (fig. 2) which allows a good separation of outer and inner mitochondrial membranes each being contaminated by the other to an extent of less than 5%. This can also be demonstrated in electron micrographs. As a marker for the outer membrane we used monoamine oxidase and for the inner membrane succinic dehydrogenase. One can see that proline oxidase not only parallels SDH in fractionated extraction procedure but also closely parallels SDH on the density gradient thus proving its tight binding to the inner mitochondrial membrane.

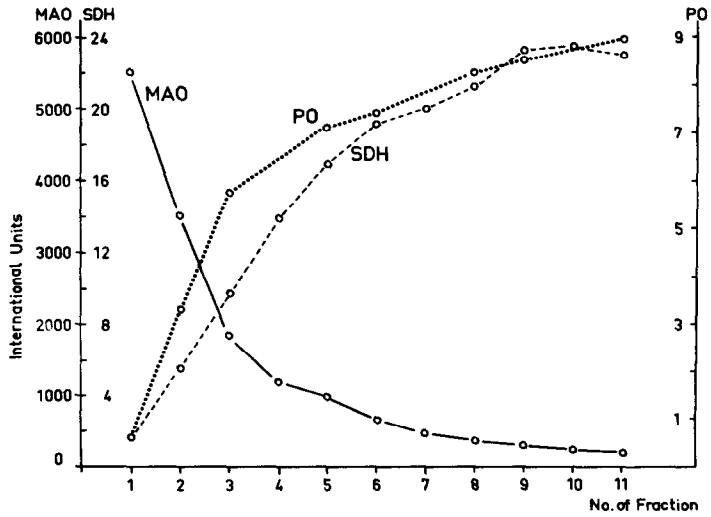

Fig. 2. Density centrifugation on a discontinuous sucrose gradient of mitochondria treated with swelling-shrinking and mild sonication. Marker enzymes expressed in terms of specific activity.

Table 2
Activity of liver enzymes after homogenisation of rat liver and centrifugation at 12 000 × *g* for 10 min.

	Glutamate dehydrogenase	Malate dehydrogenase	Proline oxidase	Δ-pyrroline-5-carboxylic acid dehydrogenase
Supernatant	7%	65%	2%	55%
Pellet	93%	35%	98%	45%

Table 3
Distribution of mitochondrial enzymes after 20 min incubation of mitochondria at 4°C under constant stirring in 0.44 M sucrose medium containing 2.1 mg digitonin per 10 mg protein and 10 min centrifugation at 10000 × *g*.

		MDH	GLUDH	PCDH	ADK	SDH	MAO
		Activity in % of total					
Digitonin:	Supernatant	19	20	18	98	4	55
	Sediment	81	80	82	2	96	45
Control:	Supernatant	2	3.5	2	3	2	3
	Sediment	98	96.5	98	97	98	97

To answer the question as for the location of Δ-pyrroline-5-carboxylic acid dehydrogenase within the mitochondrion we applied the digitonin method of Schnaitman [9] as modified by Brdiczka and coworkers [8] allowing the separation of enzymes of the matrix and the intercristal space. We found Δ-pyrroline-5-carboxylic acid dehydrogenase to behave like MDH and GLUDH (table 3) being located in the matrix in contrast to adenylate kinase which is located in the intercristal space [8].

4. Discussion

The entire location of proline oxidase in the inner membrane of rat liver mitochondria indicates that the degradation of proline is exclusively bound to the mitochondrion while the next step, the degradation of Δ-pyrroline-5-carboxylic acid can take place in the matrix as well as in the cytosole. The presence of Δ-pyrroline-5-carboxylic acid dehydrogenase in the cytosole could be an artefact if the enzyme would leak out of the particles. We therefore washed mitochondria 5 times and could not find a release of the enzyme. Also after the treatment with digitonin PCDH was released to no larger extent than MDH and GLUDH.

The difference in the location of these two enzymes made us consider that the presence of PCDH in the cytosole might be part of a system regenerating glutamate to Δ-pyrroline-5-carboxylic acid in a backward reaction. We investigated this but could not find a backward reaction neither in the cytosole nor in the matrix. Changes in substrate concentration, addition of DPNH, TPNH and glutamate, and changes in pH did not produce a backward reaction.

As described by Strecker [1] PCDH reacted faster with DPN than with TPN while the oxidation proceeded to the same endpoint with either pyridine nucleotide. Strecker described the speed ratio of the utilisation of DPN and TPN to be 5:1 in PCDH of ox liver. This is the same ratio we found in PCDH of the mitochondrial matrix; however in the cytosole TPN was oxidized twice as fast. The ratio of DPN and TPN was 5:2. This observation needs to be investigated further in order to determine whether two different enzymes exist: a DPN- and a TPN-specific PCDH.

Acknowledgement

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