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DISTRIBUTION OF GLUTAMINASE ISOENZYMES IN KIDNEY CELLS

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SUMMARY

The intracellular distribution of glutaminase isoenzymes has been investigated in rat kidney. It was found that phosphate-dependent glutaminase is a mitochondrial enzyme whereas phosphate-independent isoenzyme has a mainly microsomal localization. However, the possibility that phosphate-independent glutaminase is partly associated with the outer mitochondrial membrane cannot be ruled out.

On the basis of these experimental data it was concluded that the activity of the phosphate-dependent glutaminase is responsible for the ability of kidney mitochondria to respire in the presence of glutamine as substrate, whereas the activity of the phosphate-independent glutaminase is primarily in connection with the extramitochondrial processes.

INTRODUCTION

There are two known types of glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) in animal tissues. One of these isoenzymes is activated by inorganic phosphate and is called phosphate-dependent glutaminase [1], the other does not require this anion for the activation and is known as phosphate-independent glutaminase [2]. The phosphate-independent enzyme has been intensively studied and well characterized by Katunuma and his co-workers [3]. This isoenzyme, which is activated by maleate and carbonate ions, was found in different organs (kidney, liver, brain). Although the phosphate-dependent enzyme is well known as a mitochondrial enzyme [4], we have not found data directly related to its localization in rat kidney cells, whereas location of phosphate-independent glutaminase is still not well documented. According to Katunuma and co-workers [3] most of the phosphate-independent glutaminase activity was found in the mitochondrial fraction, whereas according to Horowitz and Knox [5] it is divided about equally between the mitochondrial fraction and the supernatant. By using pig kidney Crompton et al. [6]. found that it is localized exclusively in the mitochondria. The following communication represents a more detailed study regarding this problem.

MATERIAL AND METHODS

Tissue fractionation

Four adult albino rats were killed by decapitation. The kidneys were removed and homogenized in an ice-cold medium containing 0.25 M sucrose, 5 mM Tris–HCl (pH 7.4), and 1 mM EGTA. Six up-and-down strokes were done with a loose-fitting pestle of a Potter–Elvehjem homogenizer (A. H. Thomas Co., Philadelphia). The homogenized suspension was spun for 7 min at 550 $\times$ *g*. The sediment was washed twice in the same medium, being rehomogenized each time by six up-and-down strokes. Finally it was resuspended in 50 ml of the same medium. This suspension constituted the nuclear fraction. Supernatants obtained from the centrifugations were combined to make the cytoplasmic extract E. Fractionation of this extract was achieved using a Spinco Model L preparative ultracentrifuge. A "heavy" mitochondrial fraction (M), a "light" mitochondrial fraction (L), a microsomal fraction (P), and a soluble fraction (S) were isolated according to the fractionation scheme described by De Duve et al. [7]. The particulate fractions, two mitochondrial and one microsomal, were successively isolated by centrifugation at 3700 $\times$ *g* (10 min), 16 000 $\times$ *g* (10 min) and 105 000 $\times$ *g* (60 min), respectively. The "heavy" mitochondrial fraction was washed two times and the washings were combined with the first supernatant. In each case the sediments were resuspended in the sucrose medium.

Subfractionation of mitochondria

This was done by the method of Sottocasa et al. [8]. Mitochondria (about 60 mg protein) were allowed to swell at 0 °C in 5.5 ml (final volume) 10 mM Tris–phosphate (pH 7.4). After 8 min 5.5 ml of a solution containing 1.8 M sucrose and 2 mM ATP (Tris salt) was added (final concentration of sucrose was 0.9 M). 7 min after the addition of the hypertonic solution the suspension was sonicated in aliquots of 4 ml (15 s, 1.7 A, MSE 60 W sonicator) to detach the outer membrane from the contracted inner membrane. The sonicated suspension was diluted with 10 mM Tris–phosphate (pH 7.4) so that the final concentration of sucrose was 0.45 M. 16 ml of this suspension was layered over 40 ml of 1.2 M sucrose, in a 60-ml Beckman Spinco centrifuge tube and centrifuged in a SW 25 rotor at 24 000 rev./min for 3.5 h. After centrifugation the "soluble" and "light" fractions were sucked off by means of a Pasteur pipette, and the "heavy" fraction was resuspended with 0.45 M sucrose containing 10 mM Tris-phosphate buffer.

Enzyme assays

Phosphate-dependent and phosphate-independent glutaminase activities were assayed at 30 °C by measuring the NH_3 production enzymically [9] or by means of an ion-selective electrode (Electronic Instruments Ltd, Richmond, Surrey, England) [10]. The assay mixture contained: (a) for phosphate-dependent glutaminase: 100 mM potassium phosphate (or Tris–phosphate), 100 mM Tris–HCl and 40 mM glutamine. The final pH was 8.5, and the final vol. was 2 or 5 ml; (b) for phosphate-independent glutaminase: 100 mM Tris–HCl, 25 mM maleate (Tris salt), 1 mM phosphate (Tris salt), 0.1 mM KCl and 10 mM glutamine (final pH 7.4). The final vol. was 2 or 5 ml. Before incubation the material was heated 3 min at 52 °C to destroy phosphate-dependent glutaminase. Under these conditions no activity of the latter enzyme can be detected.

Glutamate dehydrogenase activity was determined spectrophotometrically at 25 °C by following the oxidation of NADH at 340 nm. The assay mixture contained (in 2.8 ml): 67 mM phosphate buffer (pH 7.4), 0.25 M NH_4Cl, 2 mM EDTA, 0.5 mg NADH(Na_2) and 0.1 % Triton X-100. The reaction was started by the addition of 5 mM oxoglutarate.

Cytochrome *c* oxidase was estimated by using a double-beam spectrophotometer from the change in absorbance at 605 minus 630 nm following the addition of sodium dithionite. 100 mM potassium phosphate (pH 7.4) was used as a buffer.

Rotenone-insensitive and rotenone-sensitive NADH–cytochrome *c* reductase were estimated at 25 °C with a spectrophotometer by measuring the change in the absorbance at 550 nm after the addition of NADH. The assay mixture contained (in 2 ml): 75 mM potassium phosphate buffer (pH 7.4), 1 mM KCN, 0.1 mM cytochrome *c*, 0.15 mM NADH and, when present, 10 μg rotenone.

Malate dehydrogenase activity was determined spectrophotometrically at room temperature by following the oxidation of NADH at 340 nm. The assay mixture contained in 2 ml: 50 mM Tris–HCl (pH 7.4), 10 μg rotenone, 0.1 % Triton X-100, 0.1 mM NADH and 5 mM oxaloacetate.

Glucose-6-phosphatase was determined at 37 °C according to Swanson [11]. The assay mixture contained: 0.1 M sodium citrate buffer (pH 6.5) and 25 mM glucose 6-phosphate (sodium salt). The final vol. was 1.2 ml. The inorganic phosphate liberated was estimated by the method of Berenblum and Chain [12].

Acid phosphatase activity was assayed at 37 °C. Incubations were carried out with 0.05 M β-glycerophosphate (sodium salt) as substrate, 0.05 M sodium acetate buffer, pH 5.0, and 0.1 % Triton X-100. The reaction was stopped by the addition of 1 ml of trichloroacetic acid (20 %, w/v) and inorganic phosphate was measured by the method of Berenblum and Chain [12].

Protein was determined by the biuret method as described by Gornall et al. [13].

RESULTS

1. Differential centrifugation of rat kidney homogenate

The distribution of glutaminase isoenzymes and the marker enzymes found after differential centrifugation are listed in Table I and shown graphically in Fig. 1 in the manner proposed by De Duve et al. [7].

The nuclear fraction (not shown) contained a relatively high but almost the same percentage (30–35 %) of activity in the case of all the enzymes tested. This could be explained by contamination with cell debris and a quite large proportion of unbroken cells. Glutamate dehydrogenase, acid phosphatase, glucose-6-phosphatase and rotenone-insensitive NADH–cytochrome *c* reductase were the reference enzymes for mitochondria, lysosomes, microsomes, and the outer mitochondrial membrane and microsomes, respectively. It can be seen from Fig. 1 that the distribution pattern of phosphate-dependent glutaminase is very similar to the distribution pattern of glutamate dehydrogenase although there is a slight difference. Washing of the mitochondria showed that phosphate-dependent glutaminase closely follows the distribution of glutamate dehydrogenase while the distribution of the other marker enzymes is much more different (Fig. 3).

TABLE I

DISTRIBUTION OF PROTEIN AND ENZYME ACTIVITIES IN HEAVY MITOCHONDRIAL (M), LIGHT MITOCHONDRIAL (L), MICROSOMAL (P) AND FINAL SUPERNATANT (S) FRACTIONS OF RAT KIDNEY CELLS

Enzyme activity in each fraction was calculated as a percentage of the total activity found in the cytoplasmic extract (E).

	No. of experiments	Percentage values					
		E	M	L	P	S	Recovery
Proteins	3	100	13.5	15.6	13.5	53.0	95.6
Glutamate dehydrogenase	3	100	55.2	21.3	13.2	9.1	98.8
Acid phosphatase	3	100	25.2	20.6	20.7	24.0	90.5
Glucose-6-phosphatase	3	100	3.4	41.0	51.0	0	95.4
NADH–cytochrome *c* reductase	3	100	13.5	28.0	39.0	7.9	88.4
Phosphate-independent glutaminase	3	100	7.5	26.9	39.6	16.0	90.0
Phosphate-dependent glutaminase	3	100	36.0	29.0	8.0	20.0	93.0

The distribution pattern of phosphate-independent glutaminase is the most similar to the distribution of rotenone-insensitive NADH–cytochrome *c* reductase although it is not very much different from the distribution pattern of glucose-6-phosphatase. It was found that the relative specific activity of phosphate-independent

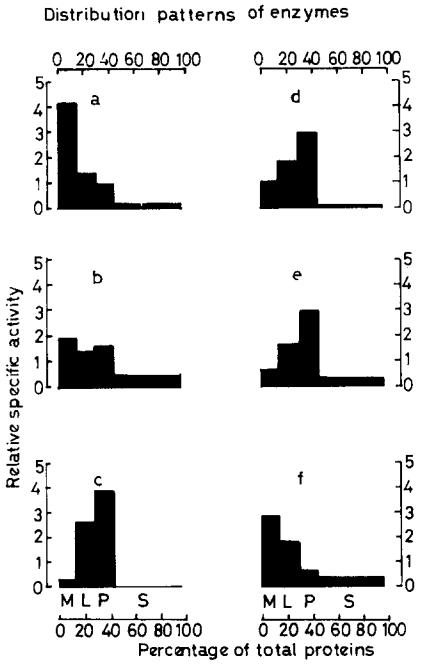

Fig. 1. Distribution patterns of enzymes: (a), glutamate dehydrogenase; (b), acid phosphatase; (c), glucose-6-phosphatase; (d), rotenone-insensitive NADH–cytochrome *c* reductase; (e), phosphate-independent glutaminase and (f), phosphate-dependent glutaminase. Abscissa, relative protein content of fractions. Ordinate, relative specific activity of fractions (percentage of total recovered activity/percentage of total recovered protein).

glutaminase in the "heavy" mitochondrial fraction is higher compared to glucose-6-phosphatase but lower compared to rotenone-insensitive NADH–cytochrome *c* reductase.

There is no possibility that phosphate-independent glutaminase is a lysosomal enzyme since its distribution pattern is quite different compared to the distribution pattern of acid phosphatase (Figs 1 and 3). In an attempt to clarify this problem the cytoplasmic extract was sonicated and centrifuged at 105 000 × *g* for 50 min. The activity of phosphate-independent glutaminase, acid phosphatase and rotenone-insensitive NADH–cytochrome *c* reductase was measured in the sediment and the supernatant fraction. While most of phosphate-independent glutaminase stayed bound to the membrane, acid phosphatase was liberated in the soluble fraction (Fig. 2).

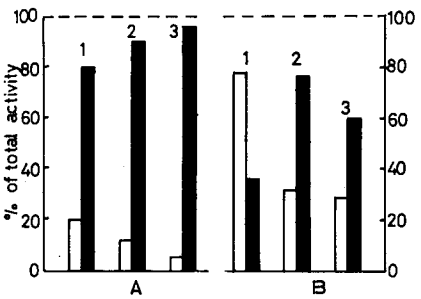

Fig. 2. Cytoplasmic extract was sonicated (B) or was not sonicated (A) with a MSE sonicator and centrifuged 60 min at 105 000 × *g*. The activity of acid phosphatase (1), phosphate-independent glutaminase (2) and rotenone-insensitive NADH–cytochrome *c* reductase (3) was measured in the sediment (■) and the supernatant (□) fraction.

2. Washing of mitochondria in order to remove microsomes

The mitochondria were sedimented firstly by centrifuging the cytoplasmic extract 10 min at 8700 × *g* and then washed three times with the same medium by centrifugation at 3700 × *g* for 10 min. All loosely packed sediments were removed with the washings. The washings were combined and centrifuged at 105 000 × *g* for 60 min. The enzyme distribution found is shown graphically in Fig. 3.

Three-times washed mitochondria were layered over 28 ml of 1.2 M sucrose in a 34-ml Spinco Model L centrifuge tube and centrifuged in a SW 25.1 rotor at 24 000 rev./min for 3 h. The distribution pattern of phosphate-independent glutaminase and the marker enzymes was the same as in Fig. 3. This isoenzyme was always between rotenone-insensitive NADH–cytochrome *c* reductase and glucose-6-phosphatase.

In a similar experiment cytoplasmic extract was centrifuged at 8700 × *g* for 15 min and the mitochondrial fraction was washed twice at 3700 × *g* for 10 min. In order to get microsomes the supernatant from the first centrifugation (without washings) was centrifuged at 105 000 × *g* for 60 min. The distribution of phosphate-independent glutaminase compared to the marker enzymes was very similar to the distribution shown in Fig. 3. However, although the microsomal fraction contained an appreciable amount of cytochrome P450 the mitochondrial fraction was virtually without this haemoprotein (Fig. 4).

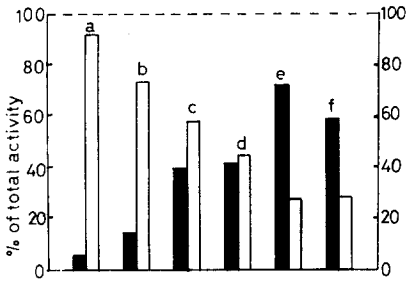

Fig. 3. Washing of rat kidney mitochondria and the distribution of enzymes: (a), glucose-6-phosphatase; (b), phosphate-independent glutaminase; (c), rotenone-insensitive NADH–cytochrome *c* reductase; (d), acid phosphatase; (e), phosphate-dependent glutaminase and (f), glutamate dehydrogenase in thrice-washed mitochondria (■) and in the washings (□). The activity of the unwashed mitochondria was calculated as 100%. The results given are the average of those obtained with six samples.

3. *Fractionation of submitochondrial particles*

Since there is a possibility that phosphate-independent glutaminase is partly associated with the mitochondria we attempted to determine its submitochondrial localization. By employing the swelling–shrinking technique of Sottocasa and collaborators [8] the outer mitochondrial membrane (light fraction), was separated from the inner mitochondrial membrane (heavy fraction) and mitochondrial matrix (soluble

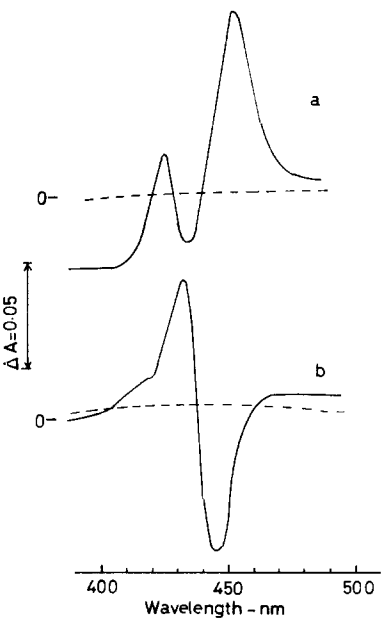

Fig. 4. Difference spectra of rat kidney mitochondria (b) and microsomes (a). In both cases cuvettes contained in 2.5 ml : 4.1 mg protein and 100 mM Tris-phosphate buffer (pH 7.4). After recording the baseline, sodium dithionite was added into both cuvettes, and then the content of the sample cuvette was gassed for 30 s with CO. The spectral changes were then recorded by using a wavelength-scanning spectrophotometer.

fraction). As the marker enzymes we used: cytochrome *c* oxidase and rotenone-sensitive NADH–cytochrome *c* reductase for the inner membrane, rotenone-insensitive NADH–cytochrome *c* reductase for the outer membrane, and malate dehydrogenase for the matrix. It can be seen from Table II that most of the phosphate-

TABLE II

DISTRIBUTION OF PROTEIN, PHOSPHATE-INDEPENDENT GLUTAMINASE (PIG) AND THE MARKER ENZYMES IN SUBMITOCHONDRIAL FRACTIONS PREPARED BY THE METHOD OF SOTTOCASA et al. [8]

Fraction	Proportion of activity recovered (%)*					
	Protein	PIG	Cytochrome oxidase	NADH–cytochrome *c* reductase		Malate dehydrogenase
				Rotenone insensitive	Rotenone sensitive	
Soluble	26	11.5	0	45	3	31
Light	10	59.0	7.1	54	3	7
Heavy	55	22.5	83.0	21	100	59
Recovery	91	93.0	90.1	90	106	97

* Compared to untreated mitochondria.

independent glutaminase activity was found in the light fraction which according to the distribution of the reference enzymes corresponds to the outer mitochondrial membrane. If the specific activity of phosphate-independent glutaminase is expressed on a protein basis then it becomes obvious that the enzyme may belong to the outer mitochondrial membrane only.

DISCUSSION

There is no doubt that rat kidney phosphate-dependent glutaminase is a mitochondrial enzyme like in other tissues so far investigated [4, 14]. It is interesting, however, that phosphate-independent glutaminase is mainly a microsomal enzyme since it was usually assumed that glutaminase is localized in the mitochondria. The possibility still exists that phosphate-independent glutaminase is partly associated with the outer mitochondrial membrane. This dilemma is based on the finding that the distribution of phosphate-independent glutaminase, although similar, does not follow exactly the distribution of either rotenone-insensitive NADH–cytochrome *c* reductase or glucose-6-phosphatase. The difficulty in the attempt to confirm the localization of this isoenzyme in the outer mitochondrial membrane could be explained partly by the heterogeneity of the kidney mitochondria and the localization of the enzyme in the outer zone of the kidney medulla only [15].

Since the mitochondrial fraction contained no cytochrome P450 it is possible to assume that the small percentage of glucose-6-phosphatase which was found in this fraction originated from contamination of the preparation with this enzyme which was detached from microsomal membrane. In any case, if phosphate-independent glutaminase is partly a mitochondrial enzyme it may be localized in the outer mito-

chondrial membrane only. It is interesting, in connection with this, that according to Crompton et al. [6] pig kidney phosphate-independent glutaminase is bound to the inside of the inner mitochondrial membrane. This is difficult to accept because they had quite improper conditions for the phosphate-independent glutaminase assay (no maleate as activator and a high concentration of inorganic phosphate [3]). Therefore, there is reason to believe that under these conditions the activity of phosphate-dependent glutaminase interfered with the activity of phosphate-independent glutaminase. This is particularly so since the specific activity of phosphate-dependent glutaminase in pig kidney is much higher than the specific activity of the phosphate-independent isoenzyme [3]. Besides, there is no information on the percentage distribution of phosphate-independent glutaminase in different cellular fractions and how much the mitochondria were contaminated with other subcellular particles. Our preliminary investigation on pig kidney homogenate showed that the distribution of phosphate-independent glutaminase is completely different compared to the distribution of glutamate dehydrogenase and phosphate-dependent glutaminase indicating that it cannot be an enzyme bound to the inner mitochondrial membrane.

Despite recent advances in the investigation and characterization of glutaminase isoenzymes the reason for the existence of the isoenzymes and their specific role in the cellular metabolism is still unknown. Therefore, determination of their intracellular localization can throw more light on their function in the physiology of kidney cells. One of the main aims of our investigation was to answer the question, which of the two known glutaminase isoenzymes in kidney is responsible for the ability of the mitochondria to respire in the presence of glutamine as substrate. The question is even more interesting providing that it is known that glutamine is a better substrate for respiration of kidney mitochondria than glutamate [10]. Experimental results presented here and in former papers [16, 17], undoubtedly suggest that phosphate-dependent glutaminase is that enzyme which enables kidney mitochondria to respire in the presence of glutamine. Phosphate-dependent glutaminase certainly plays an important role in the oxidative and energy metabolism of these organelles, whereas the phosphate-independent glutaminase activity is associated primarily with extramitochondrial processes which remain to be explored.

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