ON THE USE OF GLUTAMATE DEHYDROGENASE AS A MITOCHONDRIAL MARKER ENZYME FOR THE DETERMINATION OF THE INTRACELLULAR DISTRIBUTION OF RAT LIVER PYRUVATE CARBOXYLASE

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1. Introduction

Glutamate dehydrogenase (GDH) is widely used as a mitochondrial marker enzyme for studying the intracellular distribution of various cellular components. Böttger et al. [1] have demonstrated with the help of this and other marker enzymes that more than 97% of rat liver pyruvate carboxylase (PC) activity is located within the mitochondria. However, Seubert and coworkers [2, 3] have presented evidence for the existence of significant extramitochondrial PC activity amounting up to a third of the total activity of rat livers. They also used GDH as a mitochondrial marker enzyme, but approached the problem in a different manner. They homogenized rat livers in various media, centrifuged at 70,000 *g* and measured the activities of GDH and PC in the respective supernatants. They also measured the total activities of these enzymes in the sonicated homogenates. Since they found a higher relative percentage (total activites = 100%) of PC than of GDH activity in the supernatants, they postulated the existence of an extramitochondrial PC. These results could also be explained by assuming that GDH is released less well from the mitochondria than PC during the homogenization of the liver. However, Seubert et al. [3] could exclude this possibility by showing that breakage of mitochondria by different methods always leads to a simultanous release of both enzymes. This result is to be expected since both enzymes are known to be located in the matrix space of the mitochondria [1, 4].

There is however another alternative explanation for Seubert's findings: One could assume that, during homogenization, equal amounts of GDH and PC are liberated into the cytosol where the GDH is unstable, whereas the PC retains its activity. The results in this communication represent strong evidence for this hypothesis. It will also be shown that by using a homogenization medium, in which GDH is stable, equal percentages of GDH and PC are found in the cytosols supporting the concept of an exclusive mitochondrial localization of PC.

2. Methods and materials

The homogenization and preparation of the cytosols from rat livers were carried out according to the method of Henning et al. [5]. Mitochondria were prepared in 0.28 M sucrose according to Johnson and Lardy [6]. Sonication of the mitochondria was performed in 0.28 M sucrose at a protein concentration of about 7 mg/ml with a Branson Sonifier model B-12 at setting 5 for 4 times during 10 sec. The sonicated mitochondrial preparation was then centrifuged at 100,000 *g* and the resulting supernatant used for the experiments of table 2. The method of Böttger et al. [1] was followed for the preparation of the extracts of the liver homogenates. Sonication of the homogenates was carried out at the same intensity as for the isolated mitochondria preparation.

PC activities were measured by the method of Henning et al. [1] by transforming the oxalacetate, formed from pyruvate, to citrate and the measuring citrate enzymatically [7, 8]. GDH determinations were carried out according to Schmidt [9]. 0.6 mM ADP [10] was added when indicated (table 1).

Table 1
Pyruvate carboxylase and glutamate dehydrogenase activities in cytosols of rat livers homogenized in various media.

	Homogenization media	State of animal	Enzyme activities (μmoles/g liver)					
			Total homogenate			Cytosol[a]		
			PC	GDH		PC	GDH	
				−ADP	+ADP		−ADP	+ADP
I	0.24 M Sucrose							
	1 mM Glutathione	fed	5.8	47.5	182	0.62 (11%)[b]	0.76 (1.6%)[b]	2.08 (1.1%)[b]
	1 mM EDTA	fed	6.8	44.9	206	1.05 (15%)	1.59 (3.5%)	3.27 (1.6%)
	Triethanolamine-HCl	fasted	8.2	90.4	236	1.82 (22%)	2.66 (2.9%)	7.27 (3.1%)
	pH 7.2 (20 mM)	fasted	8.5	65.4	236	1.65 (19%)	2.98 (4.6%)	7.27 (3.1%)
II	0.28 mM Sucrose							
	1 mM Glutathione	fed	5.8	24.6	166	0.44 (7.6%)	0.33 (1.3%)	0.48 (0.2%)
	1 mM EDTA	fed	7.1	34.2	101	0.60 (8.4%)	0.41 (1.2%)	0.37 (0.4%)
	50 mM Sodium acetate	fasted	8.2	47.3	225	1.52 (19%)	1.01 (2.1%)	1.09 (0.5%)
III	0.14 M Sucrose	fed	7.0	69.7	224	0.40 (5.7%)	3.64 (5.2%)	12.4 (5.5%)
	Potassium phosphate	fed	8.4	n.m.[c]	269	0.60 (7.1%)	n.m.	18.0 (6.7%)
	pH 7.2 (50 mM)	fasted	10.8	n.m.	321	1.60 (15%)	n.m.	40.0 (12%)
		fasted	11.8	n.m.	251	1.0 (8.5%)	n.m.	18.5 (7.3%)

[a] Preparation of cytosol according to Henning et al. [5].
[b] Numbers in brackets refer to % of total activity of the respective enzymes.
[c] n.m. means not measured.

Coenzymes and enzymes were purchased from Boehringer (Germany). All the other reagents were of the highest purity commercially obtainable. Either fed or 12–24 hr fasted (see tables) male Wistar rats (CFN COBS) from the Tierzucht Institut of the University of Zürich were used.

3. Results and discussion

The results summarized in table 1 demonstrate the reproducibility of the experiments of Seubert et al. in our hands. Media I and II are those used by Seubert's group to show the relatively higher PC than GDH activities in the cytosols. Since Seubert and coworkers had assayed the GDH without ADP, we have included the analysis both with and without this nucleotide. It is interesting to note that the GDH in medium II had lost its sensitivity towards ADP. Rather large differences in the enzyme concentrations in the cytosol were sometimes observed between various experiments with the same homogenization medium. This was found to be related to the use of tighter homogenizers or more extended homogenization periods which always led to relatively higher PC and GDH activities in the cytosols. Moreover, the cytosol activities of both enzymes were also in most cases higher in livers from fasted rats as compared to those from fed animals. This finding may well be a consequence of the observation that mitochondria from fasted rats are more fragile and therefore release more enzymes during homogenization. It is noteworthy that the ratio of GDH (assayed with ADP) to PC activity in the cytosols prepared with medium I were between 3.1 and 4.4 whereas with medium II they were only between 0.7 and 0.9.

When a different phosphate containing medium (medium III) was employed, much more GDH appeared in the cytosols than with media I and II. In the sonicated whole homogenates, on the other hand, the relative changes in GDH activities between the various media were much smaller than in the corresponding cytosols. As a result of these changes the relative percentages of total PC and GDH activities were approximately the same in the phosphate containing cytosols. This was true for the livers fed as

Table 2
Recovery of pyruvate carboxylase and glutamate dehydrogenase activities added to rat liver homogenates in various media[a].

Homogenization media	Added enzyme activities to homogenate		Found enzyme activities in cytosol		Recovery of added enzyme activities	
	Pc	GDH	PC	GDH	PC	GDH
	μmoles/g liver				%	
	–	–	0.48	3.86	–	–
I	0.54	14.1	1.15	9.82	124	42
	1.08	28.2	1.67	18.2	110	51
	1.62	42.3	2.03	21.6	96	42
	–	–	0.37	0.58	–	–
II	0.36	12.6	0.69	0.66	89	0.6
	0.72	25.2	1.16	1.45	110	3.5
	1.44	50.4	1.82	2.43	101	3.7
	–	–	0.43	15.2	–	–
III	0.73	20.8	1.13	38.5	96	112
	1.46	41.6	1.76	62.7	91	114
	2.19	62.4	3.03	85.4	119	112

[a] The added enzyme activities were obtained from mitochondria sonicated in 0.28 M sucrose (See Methods). They were added to the respective homogenization media before the livers were homogenized. The final composition of medium I, II and III was the same as described in table 1.

well as for those from fasted animals.

In the connection, some recent findings from other workers are worth mentioning. Prisco et al. [11] presented evidence that in addition to mitochondria, nuclei also contain GDH which can be measured when the nuclei are sonicated and extracted by a phosphate containing medium. This nuclear enzyme shows a different kinetic behaviour towards phosphate than the mitochondrial enzyme. On the basis of these results it could be postulated that the extra GDH we found in the cytosols prepared with phosphate containing medium is of nuclear origin. We have therefore determined the dependence of the GDH activity in the cytosols of medium III on phosphate and found that it had the kinetic properties of the mitochondrial GDH as described by Prisco et al. Moreover, King and Frieden [12] have recently reported, that pure nuclei do not contain GDH and conclude that Prisco's nuclear GDH was a result of nuclear contamination with other cellular elements.

Assuming that all of the liver GDH is indeed exclusively located inside the mitochondria, we can now consider the following hypothesis: no matter which of the three media is used, with each an equal percentage of both enzyme activities is released from the mitochondria into the cytosol. However, in the phosphate containing medium, both enzymes are stable, whereas in the cytosols of the livers homogenized with media I and II only PC is stable and some of the activity of GDH is either destroyed or bound to other particles after release from the mitochondria.

This hypothesis was tested by adding known amounts of PC and GDH activities to homogenates in the various media. The results in table 2 clearly demonstrate that the added PC activity was recovered in all media whereas with GDH, this occurred only with medium III containing phosphate. In medium I, 40–50% and in medium II more than 90% of the GDH activity were lost . These losses correspond to the results of the GDH activities reported in table 1. Whether such losses of GDH occur by inactivation or by binding to other cellular fragments is presently under investigation. So far we found that the media by themselves do not inactivate GDH of mitochondrial extracts, indicating that some other component of the liver homogenate is responsible for these losses of GDH activities.

It can be concluded from these results that only the phosphate containing medium III is suitable for the quantitative use of GDH as mitochondrial marker enzyme, whereas medium I and II are not. Furthermore, the results strongly support the view that pyruvate carboxylase is exclusively a mitochondrial enzyme in rat liver.

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