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# Identity of Cytosol and Mitochondrial Glucose 6-Phosphate Dehydrogenases

By

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(With 1 Text-figure and 4 Tables)

The enzymes having glucose 6-phosphate dehydrogenase activity exist in two distinct molecular forms: one is glucose 6-phosphate dehydrogenase located in cytosol (Glock and McLean, 1953; Newburgh and Cheldelin, 1956) and the other is the enzyme known as glucose dehydrogenase (Beutler and Morrison, 1967) or hexose 6-phosphate dehydrogenase (Ohno *et al.*, 1966) located in microsomes. They differ strikingly in kinetics, immunological properties and chromatographic and electrophoretic behaviours (Beutler and Morrison, 1967; Hori *et al.*, 1975; Kimura and Yamashita, 1972; Mandula *et al.*, 1970; Srivastava *et al.*, 1972), yet they appear to have diverged from a common ancestral molecule (Matsuoka and Hori, 1980; Mochizuki and Hori, 1976).

Physiological significance of hexose 6-phosphate dehydrogenase is still obscure, but the results of our recent studies suggest that it might be involved in microsomal steroid and drug metabolism (Hori and Takahashi, 1977; Takahashi *et al.*, 1979). When dealing with the physiological significance of glucose 6-phosphate dehydrogenase isozymes, however, we must not overlook the presence of a third isozyme in the mitochondrial fraction which was reported by several workers (Bagdasarian and Hulanicka, 1965; Baquer *et al.*, 1972; Baquer and McLean, 1972; Watanabe and Taketa, 1973; Yamada and Shimazono, 1961; Zaheer *et al.*, 1967). According to Zaheer *et al.* (1967), the mitochondrial glucose 6-phosphate dehydrogenase differs from cytosol glucose 6-phosphate dehydrogenase with respect to several parameters, such as, stability, pH optimum,  $K_m$  values, sensitivity to  $CN^-$  and  $Mg^{2+}$  and immunological response to anti-cytosol glucose 6-phosphate dehydrogenase antibody. Furthermore, Baquer *et al.* (1972) and Baquer and McLean (1972) stated that the non-oxidative enzymes as well as the oxidative enzymes of the pentose phosphate pathway exist within mitochondria, particularly in steroidogenic organs, and that their activities are latent and change independently of the activities of the cytosol counterparts. In the present studies, therefore, we examined whether a third isozyme would really exist in mitochondria and whether glucose 6-phosphate dehydrogenase activity in the

mitochondrial fraction would be ascribed to an enzyme intrinsic to mitochondria. It has been already demonstrated that mitochondrial fractions prepared by a conventional method are contaminated with hexose 6-phosphate dehydrogenase of microsomal origin and that this enzyme is recovered from the outer membrane fraction when mitochondria are subfractionated (Hori and Yonezawa, 1972). Particular attention was, therefore, paid so as to assay glucose 6-phosphate dehydrogenase and hexose 6-phosphate dehydrogenase activities differentially. This was accomplished by the use of specific antisera against these enzymes.

Just after we initiated this project, Grunwald and Hill (1976) presented evidence that glucose 6-phosphate dehydrogenase activity in rat liver mitochondria is due to contamination of both cytosol and microsomal isozymes, but not to a third isozyme. Their conclusion is supported by different approaches made in the present studies.

### Materials and Methods

*Animals* — Three months old female rats of Wistar strain were used. Some were starved for 3 days and refed on 60% sucrose–30% casein diet for 3 days as described by Tepperman and Tepperman (1958) (abbreviated as “refed” in the following description).

*Cell fractionation* — Minced livers were homogenized in 3 vol. of 0.25 M sucrose containing 10 mM iodoacetamide and centrifuged at  $600 \times g$  for 15 min. The pellet was rehomogenized and centrifuged twice as above. The pooled supernatant was then centrifuged successively at  $5,000 \times g$  for 20 min and  $144,000 \times g$  for 60 min to obtain mitochondrial and cytosol fractions, respectively. The mitochondrial fraction was washed twice with 10 vol. of 0.25 M sucrose. All procedures were performed at 4°C.

*Enzyme and protein assays* — Glucose 6-phosphate dehydrogenase activity was assayed as reported previously (Hori and Sado, 1974). When necessary, the enzyme was first treated with antiserum to hexose 6-phosphate dehydrogenase and its activity was assayed in the presence of the antiserum as described elsewhere (Takahashi *et al.*, 1979). 6-Phosphogluconate dehydrogenase was assayed with 50 mM glycine-NaOH buffer, pH 8.5, 0.6 mM NADP<sup>+</sup> and 6 mM 6-phosphogluconate in a total volume of 1 ml. Lactate dehydrogenase (Wu and Racker, 1959), glucosephosphate isomerase (Zalitis and Oliver, 1967), glutamate dehydrogenase (NAD(P)<sup>+</sup>) and isocitrate dehydrogenase (NADP<sup>+</sup>) (Sottocasa *et al.*, 1967a) were assayed as described in respective references. One unit of activity was defined as the amount of enzyme that reduced or oxidized 1  $\mu$ mol of pyridine nucleotide/min at 21°C. Succinate cytochrome *c* reductase was assayed by the method of Sottocasa *et al.* (1968b). The reduction of cytochrome *c* was followed at 550 nm. The extinction coefficient ( $E_{\text{mM}}^{1\text{cm}}$ ) of 18.5 at 550 nm for reduced minus oxidized cytochrome *c* (Omura *et al.*, 1967) was used. Monoamine oxidase was assayed

as described by Tabor *et al.* (1954). Protein was determined according to Lowry *et al.* (1951) with bovine serum albumin as a standard.

*Purification of glucose 6-phosphate dehydrogenase from mitochondrial fraction*

— All buffers used for purification of enzymes contained 1 mM EDTA and 7 mM 2-mercaptoethanol. Mitochondrial fractions were homogenized with 1 M NaCl in 20 mM triethanolamine-HCl buffer, pH 6.8 (4 ml/fraction from g liver) and centrifuged at  $16,000 \times g$  for 20 min. The supernatant was treated with ammonium sulfate and the 22–32% (w/v) precipitate was dialyzed overnight against 20 mM phosphate buffer, pH 6.0 containing 12  $\mu$ M NADP<sup>+</sup>. After removal of insoluble materials, the solution was applied on a DEAE-Sephadex column (2.2  $\times$  5 cm) equilibrated with the dialysis buffer. The column was washed with 100 ml of 0.1 M KCl in the buffer and the enzyme was eluted with 50 ml of 0.3 M KCl in the buffer. The enzyme solution was adjusted to pH 6.2 with 20 mM Na<sub>2</sub>HPO<sub>4</sub>, diluted with 2 vol. of 20 mM phosphate buffer, pH 6.2 and applied on a column of 2',5'-ADP-Sepharose 4B (1  $\times$  4 cm) equilibrated with 0.1 M KCl in 20 mM phosphate buffer, pH 6.2. The column was washed with 0.1 M KCl in 0.1 M phosphate buffer, pH 6.6 and the enzyme was eluted with 1 mM NADP<sup>+</sup> in the buffer. The yields and specific activities of the various fractions obtained during one purification procedure are summarized in Table 1. All purification steps were completed within 2 days and kinetic and immunological studies were performed immediately afterwards.

*Purification of rat cytosol glucose 6-phosphate dehydrogenase* — Several methods have been reported for purification of rat glucose 6-phosphate dehydrogenase (Holten, 1972; Matsuda and Yugari, 1967; Thompson *et al.*, 1976; Watanabe and Taketa, 1972), but none was satisfactory in our hands, so that the following method was devised: Livers of refed rats were homogenized in 9 vol. of 0.25 M sucrose and centrifuged at  $150,000 \times g$  for 40 min. The supernatant was treated with ammonium sulfate and the 22–32% (w/v) fraction was dialyzed against 20 mM KCl. The dialyzed solution was adjusted to pH 5.5 with 0.1 M acetic acid, allowed to stand for 30 min and centrifuged. The precipitate was then homogenized in 20 mM phosphate buffer, pH 6.4 and centrifuged. Cold 99% ethanol was added to the supernatant at final concentration of 25% (v/v). The solution was left at 21°C for 5 h, centrifuged to remove insoluble materials, and dialyzed against 0.1 M KCl. After adjusting pH to 5.5, the soluble fraction was placed on a CM-Sephadex C-25 column (5  $\times$  20 cm) which had been equilibrated in 0.1 M ammonium acetate buffer, pH 5.5, containing 10 mM KCl and 0.1 mM EDTA, and washed with 20 mM KCl (800 ml) in the column (ion filtration method of Kirkegaard *et al.* (1972)). The column was washed with 400 ml of 0.2 M KCl and the enzyme was eluted with 0.5 mM NADP<sup>+</sup> in 0.2 M KCl (400 ml) at a flow rate of 240 ml/h. Fractions of high enzyme activity were dialyzed against 20 mM phosphate buffer, pH 6.8 and the solution was applied to a hydroxylapatite column (3  $\times$  6 cm) equilibrated with the same buffer. After washing with the buffer,

the enzyme was eluted by a linear gradient formed from 150 ml each of 20 mM and 200 mM phosphate buffer, pH 6.8 at a flow rate of 60 ml/h. The enzyme thus purified had a specific activity of 210 at pH 10 and at 21°C, and was electrophoretically homogeneous.

*Preparation of antiserum* — The concentrated enzyme solution (1 mg/ml) was mixed with an equal volume of Freund's complete adjuvant. Two milliliters of this mixture was injected into a rabbit first subscapularly and then intramuscularly after 3 weeks. Blood was collected one week after the booster injection. Control serum was obtained from the same rabbit prior to immunization. Upon agar diffusion test, antiserum thus obtained gave a single precipitin line with both pure and crude enzyme samples, but not with hexose 6-phosphate dehydrogenase.

*Immunological test* — Ouchterlony agar diffusion was performed at room temperature in 1.5% (w/v) agar in 25 mM Tris-maleate buffer, pH 7.5, containing 0.5 mM EDTA and 50 mM NaCl.

*Electrophoresis* — Polyacrylamide gel electrophoresis was performed as described by Davis (1964) using  $0.3 \times 1.0 \times 7.0$  cm columns.

*Reagents* — Glucose 6-phosphate, NADP<sup>+</sup> and NADH were purchased from Kyowa Hakko Kogyo Co.; 6-phosphogluconate, galactose 6-phosphate, fructose 6-phosphate, isocitrate,  $\alpha$ -ketoglutarate, cytochrome *c* (Type III), pyruvate and Triton X-100 from Sigma; benzylamine from Wako Pure Chemical Industries, Ltd.; succinate from Koso Chemical Co. Ltd.; CM-Sephadex C-25, DEAE-Sephadex A-50 and 2',5'-ADP-Sepharose 4B from Pharmacia Fine Chemicals; hydroxylapatite from Seikagaku Kogyo Co. and Freund's complete adjuvant from Iatron Lab. All other reagents were commercial products of the highest grade available.

## Results and Discussion

*Properties of mitochondrial glucose 6-phosphate dehydrogenase* — The enzyme was purified about 1,300-fold from mitochondrial fractions of normal rat livers (Table 1). That this sample is free of hexose 6-phosphate dehydrogenase was proven immunologically and electrophoretically.

The effect of pH on mitochondrial glucose 6-phosphate dehydrogenase activity was the same as that on cytosol glucose 6-phosphate dehydrogenase reported previously (Hori and Sado, 1974) and optimal pH was about 10.0 (glycine-NaOH buffer,  $I=0.14$ ). The two enzymes were also indistinguishable each other with respect to the  $K_m$  values (Table 2), sensitivity to various chemicals (Table 3), and immunological response to anti-glucose 6-phosphate dehydrogenase and anti-hexose 6-phosphate dehydrogenase antisera. They were not inhibited by anti-hexose 6-phosphate dehydrogenase antiserum, but inhibited by anti-glucose 6-phosphate

dehydrogenase antiserum in a similar manner. In addition, anti-glucose 6-phosphate dehydrogenase antiserum formed a single precipitin line without spurs when tested against the two enzymes on an agar plate, but did not cross-react with purified hexose 6-phosphate dehydrogenase (Fig. 1).

Table 1. Purification of glucose 6-phosphate dehydrogenase from rat liver mitochondria.

Activity was assayed at pH 7.5 (Tris-HCl,  $I=0.07$ ) and at 21°C with 0.6 mM NADP<sup>+</sup> and 6 mM glucose 6-phosphate as substrates.

	Volume (ml)	Total activity (milliunits)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Mitochondrial fraction	22.5	733.1	39.0	0.020	100
Ammonium sulfate	5.2	448.9	7.3	0.061	61.2
DEAE-Sephadex	15.5	299.0	0.83	0.360	40.8
2',5'-ADP-Sepharose 4B	4.6	263.3	0.01	26.33	35.9

Table 2.  $K_m$  values of cytosol and mitochondrial glucose 6-phosphate dehydrogenases.

The cytosol enzyme was purified as described in "Materials and Methods". For purification of the mitochondrial enzyme, mitochondria isolated with 0.25 M sucrose were homogenized with 1.5 vol. of 1 M NaCl in 20 mM triethanolamine-HCl buffer, pH 6.8 and centrifuged at  $16,000 \times g$  for 20 min. The sediment was further treated with 1% Triton X-100 in 1 M NaCl+20 mM triethanolamine-HCl buffer, pH 6.8 at 4°C for 20 min. The enzyme was purified separately from the supernatant and the Triton X-100-treated sediment as described in "Materials and Methods".  $K_m$  values were obtained from Lineweaver-Burk plots. Glc-6-P: glucose 6-phosphate. Gal-6-P: galactose 6-phosphate. Buffers used for assays were Tris-HCl, pH 7.5 ( $I=0.07$ ) and glycine-NaOH, pH 10.0 ( $I=0.14$ ).

	Substrate	$K_m$ (mM)	
		pH 7.5	pH 10.0
Cytosol enzyme	Glc-6-P	0.026	0.32
	Gal-6-P	6.2	-
	NADP <sup>+</sup> (glc-6-P)	0.006	0.010
Mitochondrial enzyme Normal rat NaCl-solubilized	Glc-6-P	0.025	0.31
	Gal-6-P	5.4	-
	NADP <sup>+</sup> (glc-6-P)	0.006	0.010
Refed rat NaCl-solubilized	Glc-6-P	0.024	0.35
	Gal-6-P	5.9	-
	NADP <sup>+</sup> (glc-6-P)	0.006	0.011
Triton X-100-solubilized	Glc-6-P	0.026	0.35
	Gal-6-P	5.9	-
	NADP <sup>+</sup> (glc-6-P)	0.006	0.011

Table 3. Effects of chemicals on purified cytosol and mitochondrial glucose 6-phosphate dehydrogenases.

The enzymes were purified from refed rats as described in "Materials and Methods", and assayed for activity in the presence of chemicals at pH 7.5 (Tris-HCl buffer, I=0.07) and at 21°C, using 6 mM glucose 6-phosphate and 0.6 mM NADP<sup>+</sup> as substrates. In the case of NADPH, NADP<sup>+</sup> was used at 24  $\mu$ M. The results are expressed in percentage of control.

	Cytosol enzyme	Mitochondrial enzyme
Dehydroepiandrosterone, 70 $\mu$ M	21	27
MgCl <sub>2</sub> , 10 mM	109	108
CaCl <sub>2</sub> , 10 mM	115	115
KCN, 10 mM	115	116
NADPH, 96 $\mu$ M	33	37

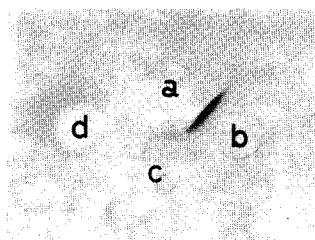


Fig. 1. Immunodiffusion on agar plate. Agar plate was stained for enzyme activity by the method previously reported (Hori and Sado, 1974). a, anti-cytosol glucose 6-phosphate dehydrogenase antiserum. b, specific antigen. c, purified mitochondrial glucose 6-phosphate dehydrogenase. d, purified hexose 6-phosphate dehydrogenase (Takahashi and Hori, 1978).

Properties of mitochondrial glucose 6-phosphate dehydrogenases which were solubilized with 1 M NaCl and 1% Triton X-100 from refed rat mitochondria were also indistinguishable from cytosol glucose 6-phosphate dehydrogenase in every respect mentioned above (Table 2). On the other hand, they differed strikingly from those of hexose 6-phosphate dehydrogenase (Beutler and Morrison, 1967; Hori and Sado, 1974). It was thus concluded that the glucose 6-phosphate dehydrogenase present in mitochondrial fractions is of the same molecular form as cytosol glucose 6-phosphate dehydrogenase, regardless of whether it is in an easily solubilized state or not.

Possibility of the presence of a third isozyme in mitochondria was thus ruled out, so that we next examined whether the mitochondrial glucose 6-phosphate dehydrogenase is inherent in mitochondria, having some physiological significance or is ascribed to a contamination of cytosol glucose 6-phosphate dehydrogenase during tissue homogenization.

*Effect of washing on mitochondrial enzyme* — As shown in Table 4, four mitochondrial marker enzymes which have been known to exist in the outer (monoamine oxidase) and inner (succinate cytochrome *c* reductase) membranes and matrix (glutamate and isocitrate dehydrogenases) were resistant to washing with 1 M NaCl, while more than 60% of glucose 6-phosphate dehydrogenase was solubilized by this treatment. This suggests that glucose 6-phosphate dehydrogenase is mostly bound to the surface of mitochondria by hydrostatic forces, this being probably of cytosol origin.

Table 4. Effects of washing on the mitochondrial and cytosol marker enzymes and glucose 6-phosphate dehydrogenase.

Mitochondria isolated with 0.25 M sucrose were treated with 1 M NaCl and centrifuged as described in the legend for Table 2. The supernatant (S) and residual (R) fractions were assayed for enzyme activities. The latter fraction was assayed in the presence of 1% Triton X-100. Glucose 6-phosphate dehydrogenase was assayed at pH 10.0 (glycine-NaOH, I=0.14). Other enzymes were assayed as described in "Materials and Methods". Results are averages of 2-4 rats.

	Cytosol activity	Mitochondrial activity after washing with			
		0.25 M sucrose		1M NaCl	
		S	R	S	R
Glucose 6-phosphate dehydrogenase (milliunits/g liver)	normal	2.5	33.1	25.9	15.8
	refed	7.9	125.9	106.7	36.2
<i>Mitochondrial marker</i>					
Monoamine oxidase ( $\Delta A/g$ liver)		0.48	1.64	0.18	1.88
Succinate cytochrome <i>c</i> reductase (units/g liver)		0.01	0.10	0.00	0.10
Glutamate dehydrogenase (units/g liver)		0.01	0.76	0.03	0.72
Isocitrate dehydrogenase (units/g liver)	11.1	0.04	0.95	0.20	0.77
<i>Cytosol marker</i>					
6-Phosphogluconate dehydrogenase (units/g liver)	4.92	0.003	0.008	0.005	0.006
Glucosephosphate isomerase (units/g liver)	37.7	0.04	0.12	0.08	0.10
Lactate dehydrogenase (units/g liver)	170.9	0.85	2.01	2.07	1.26

*Binding of cytosol enzymes to mitochondria* — Intracellular distributions of 6-phosphogluconate dehydrogenase, lactate dehydrogenase and glucosephosphate isomerase which have been known to exist exclusively in cytosol (Glock and McLean, 1953; de Duve *et al.*, 1962) are also given in Table 4. The results indicate that (1) the activities of cytosol enzymes recovered from mitochondrial fractions are negligible when compared with those in cytosol; and (2) though very low, the level of mitochondrial activity correlates with that of cytosol activity as far as the three enzymes are concerned; the higher the level in cytosol, the more was the activity recovered from mitochondria. It appears, therefore,



that very small amounts of cytosol enzyme might be incorporated into mitochondrial fractions (possibly through binding on the surface of mitochondria) during homogenization. Probably, the same might happen in the case of glucose 6-phosphate dehydrogenase.

*Effects of various substances on the solubilization of mitochondrial glucose 6-phosphate dehydrogenase* — The mitochondrial fraction isolated with 0.25 M sucrose was treated with 20 mM triethanolamine-HCl buffer, pH 6.8, containing 0.25 M sucrose plus one of the following substances; KCl, NaCl, MgCl<sub>2</sub>, CaCl<sub>2</sub>, phosphate (up to 0.2 M), AMP, ADP, ATP, CTP, GTP, UTP, NAD<sup>+</sup>, NADP<sup>+</sup>, NADH, NADPH (1–2 mM), cAMP (5  $\mu$ M), glucose 6-phosphate, glucose 1-phosphate, (0.3–0.5 mM). After centrifuging at  $16,000 \times g$  for 20 min, the supernatants were assayed for glucose 6-phosphate dehydrogenase activity.

Most effective solubilization was observed with inorganic ions; i.e., when expressed in percentage of control (activity solubilized with 1 M NaCl=100), 0.2 M NaCl or KCl caused 70% solubilization. While the same extent of solubilization was achieved by 0.1 M MgCl<sub>2</sub> or CaCl<sub>2</sub> and 0.16 M phosphate. This suggests that the effect of inorganic ions simply depends on the ionic strength. None of the other substances solubilized the enzyme above this level even when used in combination with the inorganic salts. In view of the above findings, it is concluded that the mitochondrial location of glucose 6-phosphate dehydrogenase is probably artefactual, having no physiological importance.

It is not easy to speculate the reason why Zaheer *et al.* (1967) and Watanabe and Taketa (1973) found the mitochondrial and cytosol enzymes different from each other. Their immunological data that antibody to the cytosol enzyme did not cross-react with the mitochondrial enzyme imply that their mitochondrial enzyme was hexose 6-phosphate dehydrogenase, because our data show that there is no such enzyme in the mitochondrial fraction that has a glucose 6-phosphate dehydrogenase activity, yet does not cross-react with either anti-glucose 6-phosphate dehydrogenase antibody or anti-hexose 6-phosphate dehydrogenase antibody. However, the kinetic properties reported by Zaheer *et al.* (1967) are apparently different from those reported on purified hexose 6-phosphate dehydrogenase (Grunwald and Hill, 1976; Hori and Sado, 1974). Their kinetic and immunological data are, therefore, contradictory.

### Summary

Glucose 6-phosphate dehydrogenase was purified 1,300-fold from rat liver mitochondria and its properties were examined. As a result, the enzymes solubilized with 1 M NaCl and 1% Triton X-100 were both indistinguishable from the enzyme located in cytosol with respect to *K<sub>m</sub>* values, pH optimum, sensitivity to several chemicals and immunological response to anti-cytosol glucose 6-phosphate dehydrogenase antibody.

Effects of washing, some nucleotides, sugar phosphates and inorganic ions on the solubilization of mitochondrial glucose 6-phosphate dehydrogenase were also investigated, but no positive proof was obtained that the enzyme is intrinsic to mitochondria.

It was thus concluded that mitochondrial location of glucose 6-phosphate dehydrogenase is artefactual, due to a contamination of cytosol enzyme occurred during homogenization, and is not ascribed to a distinct isozyme as predicted by some workers.

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