Biochem. J. (1976) **159**, 683–687
Printed in Great Britain

Characterization of the Glucose 6-Phosphate Dehydrogenase Activity in Rat Liver Mitochondria

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(*Received* 2 *August* 1976)

Glucose 6-phosphate dehydrogenase activity in rat liver mitochondria can be released by detergent. The released activity is separated by chromatography into two peaks. One peak has the kinetic behaviour and mobility similar to the soluble sex-linked enzyme, whereas the other peak is similar to the microsomal hexose 6-phosphate dehydrogenase. There is no evidence for the existence of a new glucose 6-phosphate dehydrogenase activity in rat liver mitochondria.

Two glucose 6-phosphate dehydrogenase activities have been described and characterized in mammalian tissues: a sex-linked glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (Bonsignore & De Flora, 1972) found in the cytosol from many sources, and an autosomally inherited enzyme, hexose 6-phosphate dehydrogenase, located primarily in the microsomal fraction of numerous tissues and animals (Mandula *et al.*, 1970). The latter is identical with glucose dehydrogenase (EC 1.1.1.47) (Beutler & Morrison, 1967).

In rat liver, a third glucose 6-phosphate dehydrogenase activity was described by Zaheer *et al.* (1967). It was located in the mitochondrial fraction, was found in sizeable amounts after a multi-step solubilization procedure, and was not inactivated by an antiserum made against partially purified soluble liver glucose 6-phosphate dehydrogenase. We have repeated the fractionation and solubilization procedures used by Zaheer *et al.* (1967) to further investigate the kinetic properties of the mitochondrial activity and to compare these properties with those of rat liver soluble glucose 6-phosphate dehydrogenase and microsomal hexose 6-phosphate dehydrogenase.

Materials and Methods

Animals

Sprague–Dawley albino female rats (2–3 months old) were killed by cervical dislocation and decapitation, after starvation for 18h.

Cell fractionation

Fractionation closely followed the procedure of Zaheer *et al.* (1965, 1967). Unless noted, all procedures were performed at 4°C. Livers were minced and homogenized for 2min at low speed in buffer containing 0.15M-KCl, 2.7mM-EDTA, 7mM-2-mercaptoethanol and 10μM-$NADP^+$, adjusted to pH7.4 with $NaHCO_3$ (1:4, w/v). EDTA, $NADP^+$ and 2-mercaptoethanol were included in the buffers as recommended by the World Health Organization (1967). The homogenate was centrifuged at 700*g* for 10min. The resulting pellet, the crude nuclear fraction, was rehomogenized and then washed three times. The pooled supernatants were centrifuged at 5000*g* for 20min. The 5000*g* pellet, the mitrochondrial fraction, was washed three times. The pooled supernatants were centrifuged at 78000*g* for 45min. The resulting pellet, the microsomal fraction, was washed once. The pooled 78000*g* supernatants comprised the soluble fraction.

Solubilization of particulate glucose 6-phosphate dehydrogenase activity

The crude particulate fractions were made 0.5% in Triton X-100, mixed immediately, incubated for 15min at 4°C, and centrifuged at 35000*g* for 1h. Additional Triton X-100 (1–2%) for up to 2h did not release any further activity.

Freezing was done at −18°C and thawing at 4°C. For ultrasonication, a Bronwill Scientific III sonicator (20kHz) with a 9mm-diameter probe was used at several different power settings and for various times. The samples were cooled in an ethanol/ice bath during sonication. After treatment, the fractions were centrifuged at 35000*g* for 1h.

Partial purification of glucose 6-phosphate dehydrogenase activity in crude cellular fractions with DEAE-cellulose

After solubilization, the cell fraction of interest was dialysed overnight against a large volume of 0.02M-potassium phosphate buffer, pH6.3, con-

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taining 2.7mM-EDTA, 7mM-2-mercaptoethanol, 10μM-$NADP^+$ and 0.2% NaN_3 (buffer A), and loaded on to an equilibrated DEAE-cellulose column (16cm×2cm) (Whatman DE52 microgranular). Activities were eluted by a potassium phosphate step gradient of 0.02, 0.04, 0.12, 0.20 and 0.50M. The protein elution profile was obtained by measuring the turbidity at 400nm of a sample of each fraction precipitated with 5% (w/v) trichloroacetic acid (Sadgopal & Bonner, 1970). Glucose 6-phosphate dehydrogenase activity was measured by the standard method (see under 'Enzyme assays') except that Mg^{2+} was omitted. The active fractions in each peak were pooled and concentrated by adding $(NH_4)_2SO_4$ saturated at 4°C (pH7) to the desired concentration and stirring slowly at 4°C for 30min. Pellets were obtained by centrifuging at 20000g for 15min. The pellets were suspended in a minimum volume of 0.1M-Tris/HCl, pH8.0, containing 2.7mM-EDTA, 7mM-2-mercaptoethanol and 10μM-$NADP^+$ (buffer B). In mitochondrial, microsomal and soluble fractions, the 30–55%-satd.-$(NH_4)_2SO_4$ pellet contained the activity.

Partial purification of glucose 6-phosphate dehydrogenase activity in the soluble fraction with gel chromatography

The soluble fraction (78000g supernatant) was dialysed overnight against buffer A, and then passed through a DEAE-cellulose column (7.0cm diam.×2cm, in a Buchner funnel) (World Health Organization, 1967). The resin was washed several times with buffer A. Glucose 6-phosphate dehydrogenase activity was then eluted with buffer A′ (identical with buffer A except the potassium phosphate concentration was 0.5M) and concentrated by a 35–55%-satd.-$(NH_4)_2SO_4$ precipitation. The sample was loaded on to an equilibrated Sephadex G-200 column (53cm×3cm; V_0 = 90ml) and eluted at a flow rate of 7.2ml/h with a 20cm head of buffer B. The active fractions, which were in a single peak, were concentrated by a 35–55%-satd.-$(NH_4)_2SO_4$ precipitation, and the final pellet was suspended in a minimum volume of buffer B.

All partial purifications were completed 3–4 days after death of the rats. Kinetic properties were investigated immediately afterwards. Protein was determined several times during the purification by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Enzyme assays

Samples were prepared for enzyme assay by dialysis overnight against 20 or more volumes of buffer B. All assays were performed in duplicate at room temperature (23°C). In the crude fractions, the activity was determined at 340nm by using the four-cuvette system of Beutler (1971) to correct for 6-phosphogluconate dehydrogenase activity. The reaction volume was 0.5ml, and included 0.05M Tris/HCl, pH8.0, 10mM-$MgCl_2$ and 0.5mM-$NADP^+$. pH–activity curves were obtained by using Tris/glycine/potassium phosphate buffer (Kirkman *et al.*, 1964). K_m values were obtained by a least-squares fit of the Lineweaver–Burk plots. A unit of dehydrogenase activity was defined as the amount of enzyme that catalyses the reduction of 1μmol of $NADP^+$/min at pH8.0 and 23°C.

Substrates and cofactors

All substrates were from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium and potassium salts of substrates were used. Galactose 6-phosphate and and 2-deoxyglucose 6-phosphate were extracted with chloroform three times to remove contaminating ethanol.

Haemoglobin determinations

Haemoglobin was determined by the benzidine method (Crosby & Furth, 1956). The haemoglobin standard of 20mg/100ml was prepared by Drabkin's technique (van Kampen & Zijlstra, 1961).

Results and Discussion

Intracellular glucose 6-phosphate dehydrogenase distribution

Activity in the various fractions after Triton treatment was determined by using 1mM-glucose 6-phosphate as substrate. In three preparations 91.6±2.7% of the total activity was found in the supernatant, and 1.9±0.85% in the microsomal, 2.0±1.0% in the mitochondrial and 4.5±1.2% in the nuclear fractions. These distributions agree with comparable data from Zaheer *et al.* (1967), and from Horne & Nordlie (1971) after correction for their fewer washes and low substrate concentration. The supernatant of the third mitochondrial fraction wash contained no activity.

Maximizing mitochondrial activity

We were unable to increase the mitochondrial activity in any samples in four separate preparations by combining the techniques of Triton solubilization, freezing and thawing, and sonication, as used by Zaheer *et al.* (1967). We found that freezing and thawing alone or 0.5% Triton alone released the maximum activity. The amount of activity existing in the mitochondrial fraction after any series of treatments never accounted for more than 4% of the total cellular activity, and never reached the 20%

Table 1. *K_m values (mM) with NADP$^+$ as cofactor*

K_m values were determined at room temperature by using at least five different substrate concentrations. All assays were done in duplicate. The reaction mix included 0.05M-Tris/HCl buffer, 10mM-$MgCl_2$ and 0.5mM-NADP$^+$. The 'soluble preparation' was the Sephadex G-200-purified fraction. References: [a], De Flora *et al.* (1968), Yoshida (1966); [b], Beutler & Morrison (1967).

(*a*)

	Human erythrocyte glucose 6-phosphate dehydrogenase[a]	Soluble preparation		0.5M-Phosphate-buffer-eluted fraction		
				Microsomal	Mitochondrial	
Substrate pH...	8	7.8	9.5	7.8	7.8	9.7
Glucose 6-phosphate	0.039	0.04	0.8	0.04	0.06	0.6
Galactose 6-phosphate	8.0, 2.3	5	>10	3	5	>10

(*b*)

	Hexose 6-phosphate dehydrogenase[b]		0.04M-Phosphate-buffer-eluted fraction			
			Microsomal		Mitochondrial	
Substrate pH...	7.1	9.6	7.8	9.4	7.8	9.6
Glucose 6-phosphate	<0.005	0.1	<0.005	0.1	<0.005	0.02
Galactose 6-phosphate	0.007	0.5	0.009	0.7	0.019	0.4

level, as one sample described by Zaheer *et al.* (1967) did.

Possible sources of glucose 6-phosphate dehydrogenase activity in the mitochondrial fraction

Given that mitochondrial activity represents only a small percentage of the total cellular activity, we wished to know if the activity represented contamination from other sources or a unique enzyme. Three sources of contamination seemed possible: (1) erythrocyte glucose 6-phosphate dehydrogenase; (2) rat liver soluble glucose 6-phosphate dehydrogenase; (3) rat liver microsomal hexose 6-phosphate dehydrogenase.

To estimate contamination from the first source, the haemoglobin content of the fractions and the whole cell extract was measured. If erythrocyte glucose 6-phosphate dehydrogenase was distributed in the fractions in a manner identical with haemoglobin, it could be calculated on the basis of an estimate of 8 units/g of haemoglobin (Dittmer, 1961) that erythrocyte glucose 6-phosphate dehydrogenase would account for less than 4% of the activity in all but the nuclear fraction. If all erythrocyte glucose 6-phosphate dehydrogenase were released into the soluble fraction, it would still only account for less than 6% of this fraction's activity.

Separation of hexose 6-phosphate dehydrogenase and glucose 6-phosphate dehydrogenase activities in the crude cellular fractions

In order to study the kinetic parameters of hexose 6-phosphate dehydrogenase and glucose 6-phosphate dehydrogenase, we purified the enzymes from the microsomal fraction and from the soluble fraction respectively. The total purification of the soluble enzyme, by using the Sephadex purification scheme, was 70-fold; that of the microsomal enzyme was 20-fold. The activity from the 0.04M-phosphate buffer elution step of the DEAE-cellulose column loaded with crude microsomal fraction was used in determining the properties of hexose 6-phosphate dehydrogenase. This enzyme was able to use NAD$^+$ as well as NADP$^+$ as cofactor; glucose 6-phosphate dehydrogenase was able to use only NADP$^+$. Glucose was a substrate of hexose 6-phosphate dehydrogenase only, and the microsomal enzyme had a greater relative activity with galactose 6-phosphate and 2-deoxyglucose 6-phosphate as substrates. K_m values differed strikingly (Table 1) and $V_{max.}$ was reached at lower substrate concentrations when the microsomal enzyme catalysed the reaction. The two enzymes also had quite different pH–activity curves when glucose 6-phosphate was used as substrate and NADP$^+$ as cofactor. These data are in good agreement with previously reported properties of hexose 6-phosphate dehydrogenase (Beutler & Morrison, 1967) and glucose 6-phosphate dehydrogenase (De Flora *et al.*, 1968; Yoshida, 1966).

The soluble fraction activity was eluted from the DEAE-cellulose column in a single peak at 0.5M-phosphate buffer. The microsomal fraction, containing some contaminating glucose 6-phosphate dehydrogenase, had 75% of its total activity eluted at 0.04M-phosphate buffer (Fig. 1). It was hoped that, if there was a unique mitochondrial enzyme, it would be eluted at a different phosphate concentration. However,

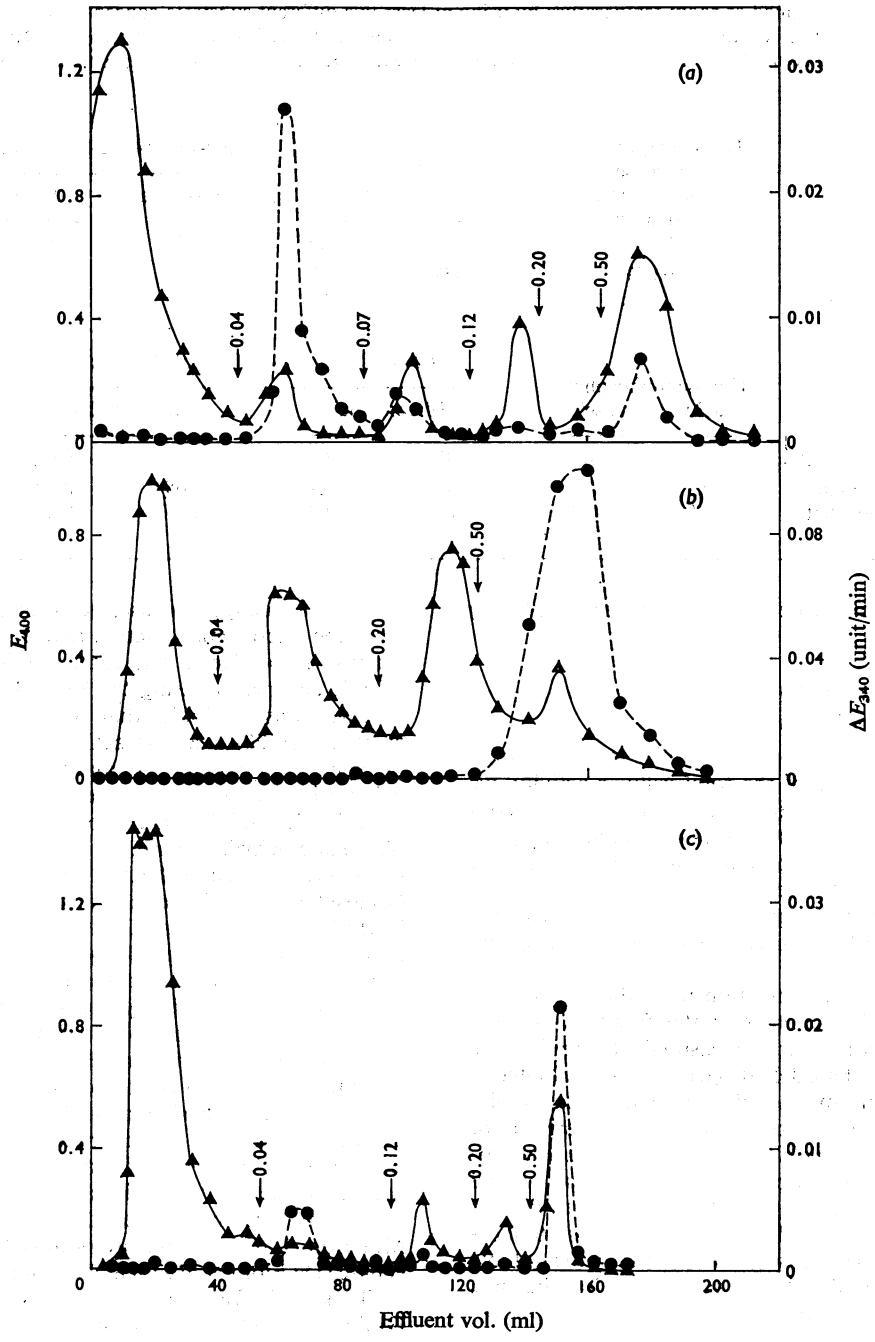

Fig. 1. *Elution profiles of the microsomal fraction* (*a*), *the soluble fraction* (*b*) *and the mitochondrial fraction* (*c*)

The initial step (0 ml) was 0.02 M-phosphate buffer. The values above the arrows indicate the phosphate buffer concentration (M). ▲, Protein elution profile; ●, glucose 6-phosphate dehydrogenase activity elution profile.

peaks of activity in the mitochondrial preparation were detected only in the 0.04M and 0.5M steps. Most of the mitochondrial activity was eluted with 0.5M-phosphate (Fig. 1). Recovery of the activity was 97, 86 and 98% on the mitochondrial, microsomal and soluble runs respectively, so it is unlikely that a more anionic enzyme exists or that an enzyme was denatured during the runs.

The kinetic properties, substrate and cofactor affinities and pH optima of all elution peaks were investigated after $(NH_4)_2SO_4$ fractionation and concentration. The activity in the 0.5M-phosphate-buffer-eluted fraction from mitochondrial, microsomal and soluble fractions resembled that of glucose 6-phosphate dehydrogenase in all respects. The 0.04M-phosphate-eluted activity of the mitochondrial preparation demonstrated that this activity was due to hexose 6-phosphate dehydrogenase. Table 1 compares the known K_m values for the two enzymes with those obtained for the various DEAE-cellulose-separated activities.

From our experiments with female rat liver (ion-exchange chromatography, pH, K_m, substrate and cofactor specificity) the enzyme activity found in the cell fractions can be explained by the differential distribution of the two well-known enzymes, glucose 6-phosphate dehydrogenase and hexose 6-phosphate dehydrogenase. No evidence was found for the existence of a distinct activity in the mitochondrial fraction.

Zaheer *et al.* (1967) found what they considered to be a new activity in rat liver mitochondria. but they did little to characterize it kinetically. They were unable to inhibit mitochondrial glucose 6-phosphate dehydrogenase activity with antibody to the soluble enzyme, and Watanabe & Taketa (1973) found no precipitin lines in the Ouchterlony test. However, the latter did not state how they solubilized their mitochondrial enzyme. Possibly these mitochondrial preparations contained other proteins which interfered with the antibody, or were heavily contaminated with microsomal hexose 6-phosphate dehydrogenase. In our experiments, approximately one-eighth of our mitochondrial activity was due to hexose 6-phosphate dehydrogenase activity, suggesting some microsomal contamination.

The two activities in the mitochondrial fraction could not be washed out, but could only be released by freezing and thawing or detergent treatment. Thus the mitochondrial activity must have been enclosed or membrane bound. Mitochondrial-size cell fragments with enclosed cell sap might well be formed by pinocytosis during the initial homogenization. de Duve (1967) has suggested that similar experimentally created fragments may account for the reports of brain mitochondrial glycolytic enzymes.

This work was supported by U.S.P.H.S. grant no. 1RO2CA13053 from the National Cancer Institute and grant no. 7RO1GM20930 from the Institute of General Medical Sciences.

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