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Purification and Properties of Microsomal Glucose 6-Phosphate Dehydrogenase (Hexose 6-Phosphate Dehydrogenase) of Rat Liver

By

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(With 7 Text-figures and 6 Tables)

Introduction

The enzymes having glucose 6-P dehydrogenase activity exist in soluble and particulate-bound forms in mammalian livers; the former is located in cytosol (Glock and McLean 1953, Newburgh and Cheldelin 1956) has a narrow substrate specificity, is sensitive to SH-inhibitors and dehydroepiandrosterone and is inducible with estradiol (Hori and Matsui 1967, 1968), while the latter is in microsomes, has a broad substrate specificity, is resistant to the inhibitors and the hormone and is not inducible with estradiol (Beutler and Morrison 1967, Hori and Matsui 1967, 1968, Mandula *et al.* 1970, Ohno *et al.* 1966, Shaw and Koen 1968). The cytosol enzymes are probably homologous with the sex-linked enzyme of human erythrocytes and the microsomal enzymes with the autosome-inherited enzymes of deer mouse and house mouse (Ohno *et al.* 1966, Shaw and Koen 1968). Ohno *et al.* (1966) designated this microsomal enzyme as hexose 6-P dehydrogenase in order to distinguish from the cytosol enzymes. Having studied in detail the properties of hexose 6-P dehydrogenase with microsomal fractions of mouse liver, Beutler and Morrison (1967) found that the enzyme was active on deoxyglucose 6-P and glucose as well as glucose 6-P and galactose 6-P in the presence of either NADP or NAD, and concluded that the enzyme might be identical with the microsomal enzyme previously reported as glucose dehydrogenase (β-D-glucose:NAD (P) oxidoreductase, EC 1.1.47) (Brink 1953, Harrison 1931, Metzger 1964, 1965, Strecker and Korkes 1952).

In electrophoretic studies of glucose 6-P dehydrogenases from a wide variety of animals, we have previously found that the enzymes from different animals have markedly different substrate specificity (Kamada and Hori 1970). The enzymes were therefore tentatively classified into three major types; type I includes the enzymes having substrate specificity similar to human erythrocyte glucose 6-P

dehydrogenase and type III, those having substrate specificity similar to the so-called hexose 6-P dehydrogenase, while type II includes the enzymes having the substrate specificity intermediate between type I and type III. Subsequent works on coenzyme specificity showed that some type II enzymes as well as type III enzymes were active also on NAD, while other type II enzymes as well as type I enzymes were active only on NADP (Mochizuki and Hori 1973). A hypothesis was then proposed that type II and III enzymes might have evolved from type I enzymes (Kamada and Hori 1970).

It is however evident that electrophoretic analysis of the enzymes cannot provide accurate data on their properties. We have therefore recently initiated a series of studies in which the enzymes are purified for detailed characterization from several species which are thought to be important from a standpoint of glucose 6-P dehydrogenase evolution, in a hope to obtain evidence on the above hypothesis.

Since we have been using the rat enzymes as the reference with which to compare the enzymes from other species, an attempt was first made to purify and to characterize the microsomal enzyme as well as the cytosol enzyme from rat liver which is dealt with in the present communication.

Materials

Rats used were adult males of Wistar strain. Chemicals were obtained from the following commercial sources; glucose 6-P, galactose 6-P, 2-deoxyglucose 6-P, gluconate 6-P, NADP, NAD, phospholipase C (type I), rabbit muscle phosphorylase a, beef liver catalase, rabbit muscle aldolase and bovine albumin from Sigma, hydroxylapatite from Serva, DEAE-Sephadex (A-50) and CM-Sephadex (C-50) from Pharmacia and CM-cellulose from Whatman. Dehydroepiandrosterone was donated by Teikokuzoki. All other reagents were commercial products of the highest grade available.

Methods

Enzyme assay

Reaction mixture contained in a total volume of 1.0 ml; 0.7 ml of glycine-NaOH buffer (I=0.2, pH 9.0–11.2) or Tris-HCl buffer (I=0.1, pH 7.0–8.9); NAD or NADP, 0.6 μmoles; enzyme, up to 50 μl; and substrate, 6 μmoles.

Reaction was started by adding substrate and the rate of coenzyme reduction was followed spectrophotometrically at 340 nm and 21±1°C during 5 min in a Hitachi 101 spectrophotometer using 1-ml cuvettes with a 1-cm light path. The rate of coenzyme reduction under these conditions was linear, both with time and with enzyme concentration, when optical density changes of less than 0.10 per min were measured. A unit of activity was taken to be the amount of enzyme which reduced 1 μmole of NAD or NADP per min. Glucose 6-P dehydrogenase activity was corrected for gluconate 6-P dehydrogenase activity, when present, by the method of Glock and McLean (1953).

Protein assay

Protein was assayed by the method of Lowry *et al.* (1951) using bovine gamma globulin as standard and specific activity of enzyme was expressed as units per mg protein.

Polyacrylamide disc electrophoresis

Electrophoresis was performed by the method of Ornstein (1964) and Davis (1964) using $3\times12\times100$ mm plastic columns. After electrophoresis at 4°C in 0.38 M glycine-Tris buffer (pH 8.3) with a constant current of 3 mA per column, the gels were stained for glucose 6-P dehydrogenase activity at 37°C in 0.1 M Tris-HCl buffer (pH 7.5), 1.2 mM glucose 6-P, 0.2 mM NADP, 0.3 mM iodonitrotetrazolium violet and 0.07 mM phenazine methosulfate, or for proteins with Amido black.

Molecular weight determination

Molecular weights were estimated by electrophoresis in polyacrylamide gels with or without dodecyl sulfate according to the methods of Zwaan (1967) and of Shapiro *et al.* (1967). In both cases, gels were cast in $3\times12\times100$ mm columns and electrophoresed for 2–3h. In the case of dodecyl sulfate polyacrylamide gel electrophoresis, electrode buffer was changed every hour. Proteins used as reference were: yeast glucose 6-P dehydrogenase, dimer, 102 000 (Yue *et al.* 1967); yeast alcohol dehydrogenase, tetramer, 150 000 (Hayes and Velick 1954); rabbit muscle phosphorylase *a*, dimer, 185 000 (Seery *et al.* 1967); bovine albumin, monomer and dimer, 68 000 and 136 000 (Loeb and Scheraga 1956); beef liver catalase, tetramer, 240 000 (Samejima and Yang 1963); rabbit muscle aldolase, tetramer, 160 000 (Kawahara and Tanford 1966). Enzyme and protein bands were located with iodonitrotetrazolium violet and Amido black, respectively.

Effects of various substances

Effects of the following substances on enzyme activity were assayed by incorporating them into the reaction mixture as follows: Dehydroepiandrosterone was dissolved in acetone at a concentration of 0.1 M. Ten microliters of this solution was blowed into 10 ml of glycine-NaOH buffer, pH 10.8 or 10.0 (I=0.2) using a micropipette. The buffers thus prepared were used for assay. The final concentration of dehydroepiandrosterone in a reaction mixture was 70 μM. Glycine-NaOH buffer containing 0.1% acetone was used as control.

p-Chloromercuribenzoate was mixed with glycine-NaOH buffer (pH 10.0, I=0.2) and dissolved by addition of a minimal amount of 1 M NaOH. pH was adjusted to 10.0 with concentrated HCl and the volume was adjusted to give a concentration of 0.1 M in respect to p-chloromercuribenzoate. Ten microliters of this solution were added to reaction mixture to give a final concentration of 1 mM.

N-ethylmaleimide was dissolved in glycine-NaOH buffer (pH 10.8, I=0.2) at a concentration of 0.2 M. Ten microliters of this solution were added to reaction mixture to give a final concentration of 2 mM.

Iodoacetamide and dithiothreitol were dissolved in deionized water at concentrations of 0.3 and 0.2 M, respectively, and aliquots were added to reaction mixture to give final concentrations of 3 and 2 mM, respectively.

Phospholipase C(mg/ml of 0.1 M Tris-HCl buffer, pH 8.1), 0.6 ml, was mixed with 0.3 ml of enzyme sample and 20 μl of 0.1 M $CaCl_2$, and incubated for 30 and 60 min at 37°C. Phospholipase C was replaced by Tris-HCl buffer for control. Aliquots of the above mixture were assayed for glucose 6-P dehydrogenase activity.

$MgCl_2$ and $CaCl_2$ were added to the reaction mixture at final concentrations of 5 and 10 mM, and the enzyme activity was assayed at pH 7.5 using Tris-HCl buffer.

Effect of heat

Aliquots of enzyme solution were sealed in vials and heated at 50°C for 5, 10 and

20 min in a water bath. Heating was terminated by immersing the vials in a cold water bath (2°C). The enzyme activity of the sample thus treated was assayed and the result was expressed as percentage of the untreated control.

Results and Discussion

Purification of microsomal glucose 6-P dehydrogenase

All steps were carried out at 2–4°C. EDTA was added at a concentration of 2 mM to all the buffers used.

Cell fractionation.

Livers were rinsed in cold 0.25 M sucrose-2 mM iodoacetamide, blotted on filter papers, weighed, homogenized in about 3 volumes of sucrose and centrifuged at 750×g for 10 min. The sediment was suspended in sucrose, homogenized and centrifuged again. This step was repeated once again and the supernatants were pooled and centrifuged first at 8 500×g for 30 min to remove mitochondria and then at 50 000×g for 1 h to obtain microsome and cytosol fractions. The microsome fraction was mixed with 20 mM phosphate buffer (pH 6.4)–2 mM iodoacetamide (2 ml per fraction from g liver) and sonicated in a Kubota ultrasonicator for 30 min at 200 W.

Ammonium sulfate precipitation.

To the sonicated microsome fraction was added 1.0 vol. of 3.78 M ammonium sulfate and the mixture was stirred for 30 min at 2°C before the precipitate was collected by centrifugation. The precipitate was then dissolved in 20 mM phosphate buffer (pH 6.8)–2 mM iodoacetamide and dialyzed overnight against the same buffer. After removal of insoluble substances by centrifugation, the dialysis was completed with the aid of an Amicon hollow fiber concentrator, Model CH3 with a H1CX50 cartridge.

Batch chromatography on hydroxylapatite gel.

The solution from the previous step was mixed with hydroxylapatite gel previously equilibrated in 20 mM phosphate buffer (pH 6.8; 1 ml settled volume per solution from g liver), allowed to stand for 30 min and centrifuged to sediment the gel. The gel was washed first with 20 mM and then with 50 mM phosphate buffer (pH 6.8). After washing the gel was suspended in 0.15 M phosphate buffer (pH 6.8)–2 mM iodoacetamide and left for 45 min before separation of gel by centrifuging. This step was repeated once again. The 0.15 M phosphate buffer fractions were pooled and condensed with the Amicon hollow fiber concentrator. The condensed sample was mixed with 1.5 vol. of 3.78 M ammonium sulfate, stirred for 30 min and centrifuged. The precipitate was dissolved in 20 mM phosphate buffer (pH 6.4), and dialyzed against the same buffer.

CM-Sephadex chromatography.

The dialyzed solution was placed on a CM-Sephadex column (32×600 mm) which had been equilibrated in 20 mM phosphate buffer (pH 6.4) and the enzyme was eluted by using a linear gradient formed from 300 ml of 20 mM phosphate

buffer (pH 6.4) and 300 ml of 0.1 M Na_2HPO_4-2 mM EDTA (pH 8.4) at a flow rate of 20 ml per h (Fig. 1). Fractions of 6 ml were collected and those with high enzyme activity (about pH 6.8) were pooled. The pooled fractions were dialyzed against 20 mM phosphate buffer (pH 6.8) overnight.

DEAE-Sephadex chromatography.

After removal of insoluble materials by centrifugation the solution from the previous step was placed on a DEAE-Sephadex column (10×600 mm) which had been equilibrated in 20 mM phosphate buffer (pH 6.8). The column was washed with 100 ml of 20 mM phosphate buffer (pH 6.4) and then the enzyme was eluted by using a linear gradient formed from 100 ml of 20 mM phosphate buffer (pH 6.4) and 100 ml of 0.4 M NaCl in 20 mM phosphate buffer (pH 6.4) at a flow rate of 12 ml per h (Fig. 2). Fractions of 3 ml were collected and those with high enzyme activity were pooled.

The yields and specific activities of the various fractions obtained during one purification procedure is summarized in Table 1.

The purity of the final product was examined by polyacrylamide disc electrophoresis. As shown in Fig. 2, a single protein band which coincided well with the glucose 6-P dehydrogenase activity was observed.

Purification of cytosol glucose 6-phosphate dehydrogenase

Adult rats were fasted for 3 days and refed for 3 days on a diet containing 60% glucose and 30% casein. This dietary treatment has been reported to induce the cytosol glucose 6-P dehydrogenase in rat liver (Tepperman and Tepperman

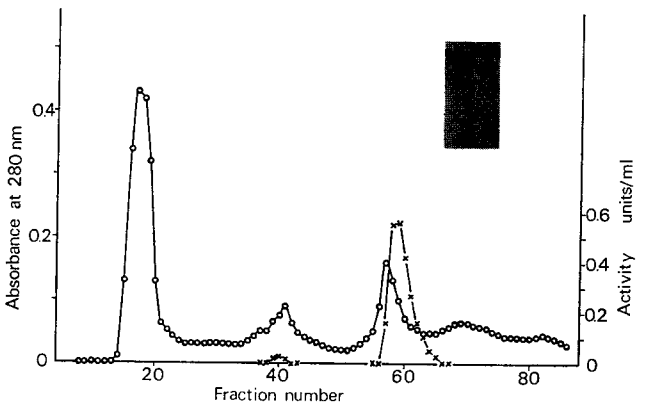

Fig. 1. CM-Sephadex column chromatography of microsomal glucose 6-P dehydrogenase. The dialyzed solution from hydroxylapatite gel chromatography was placed on a CM-Sephadex column (32×600 mm) and the enzyme was eluted by increasing pH and concentration of Na_2HPO_4. Fractions, 57–62 were pooled and dialyzed. ×—×, enzyme activity; ○—○, protein.

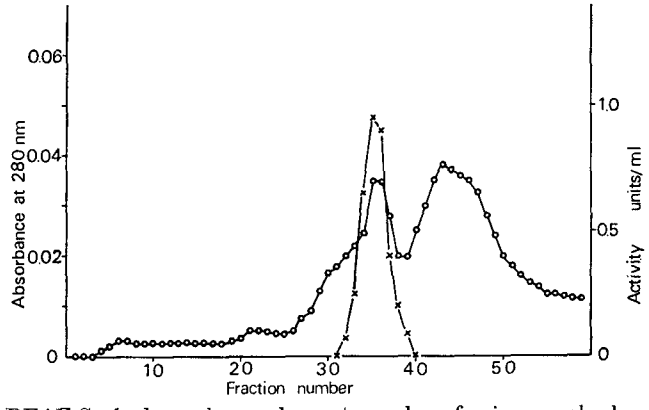

Fig. 2. DEAE-Sephadex column chromatography of microsomal glucose 6-P dehydrogenase. The dialyzed solution from CM-Sephadex column chromatography was placed on a DEAE-Sephadex column (1×60 cm) and the enzyme was eluted by increasing NaCl concentration in 20 mM phosphate buffer (pH 6.4). Fractions, 34–36 were pooled (10.0 units per mg protein) and used for analysis of enzyme properties. Zymograms show that the enzyme solution contains a single activity band (right) which accords with the protein band (left). ×—×, enzyme activity. ○—○, protein.

Table 1. Purification of microsomal glucose 6-P dehydrogenase from rat liver. Activity was assayed at pH 10.8 with glucose 6-P and NADP.

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Homogenate*	868	57.1**	17200	0.003	100
Microsome fraction	182	33.0	2680	0.012	57.8
Ammonium sulfate	144	24.4	457	0.053	42.7
Hydroxylapatite	21.1	15.0	83.2	0.180	26.3
CM-Sephadex	36.0	13.9	11.9	1.17	24.3
DEAE-Sephadex	9.0	7.52	0.75	10.0	13.2

* Homogenate was sonicated for enzyme assay.

** This is calculated by: $y=\frac{Y-0.1X}{0.94}$, $x=X-0.58y$, where X is total activity at pH 10.0; Y, total activity at pH 10.8; x, activity of cytosol enzyme at pH 10.0; y, activity of microsomal enzyme at pH 10.8. The optimal pH of the microsomal and cytosol enzymes differ distinctly, each exhibiting a considerable activity decrease at the optimal pH of the other. Taking advantage of this phenomenon, the activity of each enzyme can be estimated in a mixture of both enzymes, simply assaying the activities at both pH. Data of model experiments with the purified enzymes exactly followed the above equations, though this does not necessarily mean that the equations are also applicable to crude tissue extracts. Since there is no other method available, the above equations were tentatively adopted.

1958). All the following steps were carried out at 2–4°C. NADP was added to all the buffers used at a concentration of 12 μM. In addition, EDTA (2 mM) and β-mercaptoethanol (7 mM) were added to phosphate buffers. The following method was a modification of the method of Matsuda and Yugari (1967).

Cell fractionation.

Livers were homogenized with 9 parts of 0.25 M sucrose and centrifuged successively at 750×g and 8 500×g in order to remove tissue debris and mitochondria. The supernatant was then centrifuged at 105 000×g for 1 h to obtain cytosol fraction.

Ammonium sulfate precipitation.

The cytosol fraction was mixed with 2/3 vol. of 3.78 M ammonium sulfate. After 30 min, the precipitate was removed by centrifugation. To three volumes of the resultant supernatant were added two volumes of 3.78 M ammonium sulfate. After 30 min the precipitate was collected by centrifugation, dissolved in 20 mM phosphate buffer (pH 6.4) and dialyzed against the same buffer.

DEAE-Sephadex chromatography.

The dialyzed solution from the previous step was centrifuged and the supernatant was placed on a DEAE-Sephadex column (36×400 mm) which had been equilibratedin 20 mM phosphate buffer (pH 6.4). After washing with 200 ml of the buffer and then with 200 ml of 0.2 M NaCl in the same buffer, the enzyme was eluted at a flow rate of 30 ml per h by using a linear gradient formed from 200 ml of 0.2 M NaCl and 200 ml of 0.6 M NaCl, both in 20 mM phosphate buffer (pH 6.4) (Fig. 3). Fractions of 10 ml were collected and those with high enzyme activity were pooled.

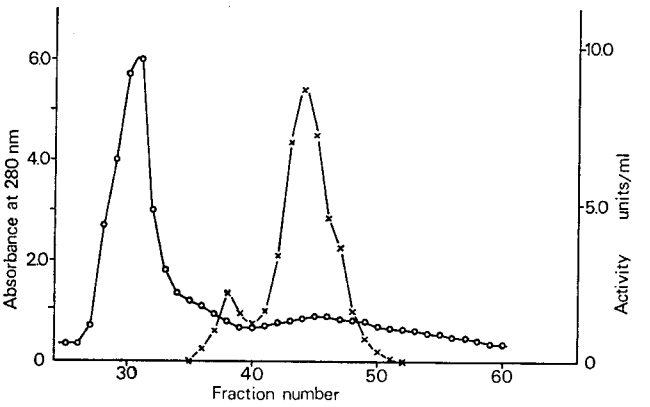

Fig. 3. DEAE-Sephadex column chromatography of cytosol glucose 6-P dehydrogenase. The ammonium sulfate precipitates from cytosol fraction were dialyzed, and placed on a DEAE-Sephadex column (36×400 mm). The enzyme was eluted by increasing NaCl concentration in 20 mM phosphate buffer (pH 6.4). Fraction, 42–47 were pooled and used for further purification. ×—×, enzyme activity; ○—○, protein.

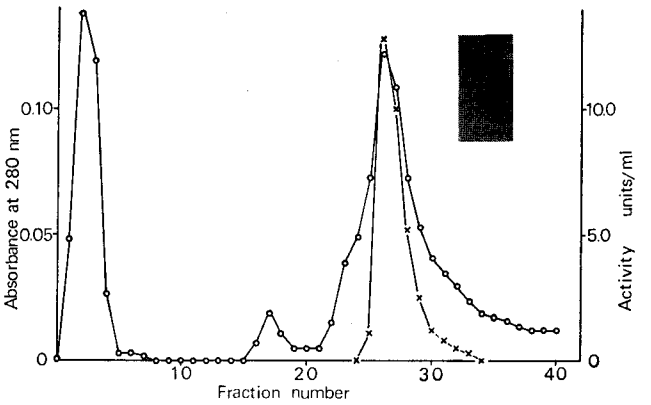

Fig. 4. CM-cellulose column chromatography of cytosol glucose 6-P dehydrogenase. The solution from the pH 5.5 treatment was placed on a CM-cellulose column (10×120 mm) and the enzyme was eluted by increasing pH and concentration of CH_3COONH_4. Fractions 26 and 27 were pooled and used for analysis. Zymograms show two major and four minor bands of activity (right) and three protein bands, one of which does not accord with activity band (left). ×—×, enzyme activity; ○—○, protein.

pH 5.5 treatment.

The pooled fractions were then treated with 1.5 vol. of 3.78 M ammonium sulfate. The precipitate was collected by centrifugation, dissolved in 10 mM CH_3COONH_4 and dialyzed against the same solution. After centrifugation, the resulting clear supernatant was adjusted at pH 5.5 with 0.1 M CH_3COOH, allowed to stand for 30 min and centrifuged to remove insoluble materials.

CM-cellulose chromatography.

The solution was then placed on a CM-cellulose column (10×120 mm) which had been equilibrated with 10 mM CH_3COONH_4 buffer (pH 5.5). After the column

Table 2. Purification of cytosol glucose 6-P dehydrogenase from rat liver. Activity was assayed at pH 10.0 with glucose 6-P and NADP.

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Cytosol fraction	313	626*	1950	0.321	100
Ammonium sulfate	44	704	563	1.25	112
DEAE-Sephadex	58	485	31	15.6	77.5
pH 5.5 treatment	16.6	427	12.4	34.4	68.2
CM-cellulose	15.4	183	1.5	122	29.2

* Activity of microsomal glucose 6-P dehydrogenase in this fraction was very small, judging from a ratio of galactose 6-P dehydrogenase activity to glucose 6-P dehydrogenase activity.

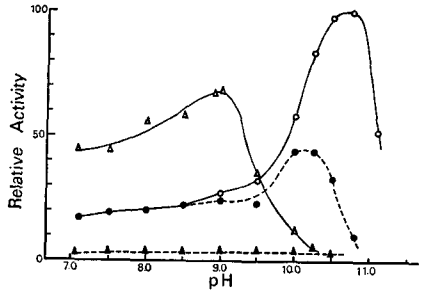

Fig.5

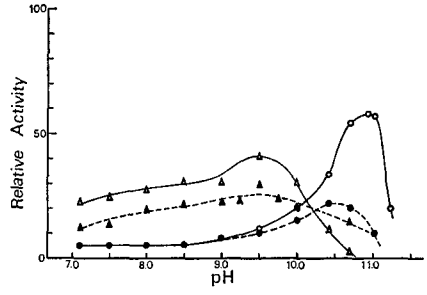

Fig. 6

Fig. 5. Effect of pH on microsomal glucose 6-P dehydrogenase activity in the presence of NADP. Concentration of substrate: glucose 6-P, galactose 6-P and deoxyglucose 6-P, 6.0 mM; glucose, 0.2 M; NADP, 0.6 mM. Activity was expressed as percentage of the activity on glucose 6-P and NADP at pH 10.8. ○—○, glucose 6-P; ●---●, galactose 6-P; △—△, deoxyglucose 6-P; ▲---▲, glucose.

Fig. 6. Effect of pH on microsomal glucose 6-P dehydrogenase activity in the presence of NAD. Substrate concentrations used were the same as in Fig. 5. NAD concentration was 0.6 mM. Activity was expressed as percentage of the activity on glucose 6-P and NADP at pH 10.8. ○—○, glucose 6-P; ●---●, galactose 6-P; △—△, deoxyglucose 6-P; ▲---▲, glucose.

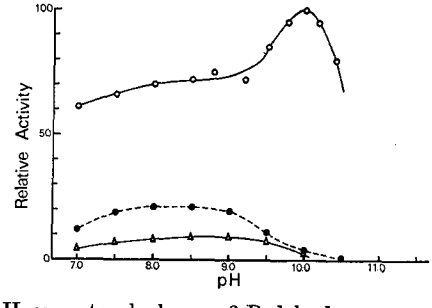

Fig. 7. Effect of pH on cytosol glucose 6-P dehydrogenase activity. Substrate and NADP concentrations were 6.0 mM and 0.6 mM, respectively. Activity was expressed as percentage of the activity on glucose 6-P at pH 10.0. ○—○, glucose 6-P; ●---●, galactose 6-P; △—△, deoxyglucose 6-P.

was washed with 100 ml of the buffer the enzyme was eluted at a flow rate of 12 ml per h by using a linear gradient formed from 200 ml of 10 mM CH_3COONH_4 buffer (pH 5.5) and 200 ml of 50 mM CH_3COONH_4 (Fig. 4). Fractions of 8 ml were collected and those with high enzyme activity (pH 5.8) were pooled and concentrated with the use of a collodion bag (Sartorius membrane filter).

The yields and specific activities of the various fractions obtained during one purification procedure is summarized in Table 2.

The purity of the final product was examined by polyacrylamide disc electrophoresis. As illustrated in Fig. 4, two major bands of the enzyme activity were observed in addition to some minor bands of less activity. The major bands correspond to the D and F bands in our previous study (Hori and Yonezawa 1972). One protein band which did not coincide with the enzyme activity was observed.

Comparison of the microsomal and cytosol glucose 6-P dehydrogenases

The pH-activity curves of the purified enzymes with various combinations of substrates and coenzymes are shown in Figs. 5–7. The optimal pH for the microsomal and cytosol glucose 6-P dehydrogenases were estimated 10.8 and 10.0, respectively, when glucose 6-P (6 mM) and NADP (0.6 mM) were used. At a physiological pH (7.5), the ratio of the activities toward glucose 6-P, galactose 6-P and deoxyglucose 6-P was 100:29:11 for the cytosol enzyme and that for the microsomal enzyme was 100:100:242, with NADP as coenzyme.

The *Km* values at pH 7.5, 10.0 and 10.8 were obtained from Lineweaver-Burk plots and are given in Tables 3 and 4.

The above data clearly show that the microsomal enzyme of rat liver is strikingly similar to the microsomal hexose 6-P dehydrogenase of mouse liver reported by Beutler and Morrison (1967) in the following respects:

1) Substantial activity was found with deoxyglucose 6-P, galactose 6-P and glucose with either NADP or NAD as coenzyme.

2) Greater activity was obtained with NADP than with NAD when galactose 6-P or glucose 6-P was the substrate, while the activity on glucose was greater with NAD than with NADP.

3) The Michaelis constant was very pH-dependent, greater for NADP than

Table 3. Substrate specificity of microsomal glucose 6-P dehydrogenase from rat liver. Assays were performed with the purified enzyme at pH 7.5, 10.0 and 10.8 at various substrate concentrations ranging above and below the *Km* values, except for glucose which did not saturate the enzyme even at 1.4 M. *V* is given as percentage of activity with glucose 6-P and NADP at pH 10.0.

Substrate	pH 7.5				pH 10.0				pH 10.8			
	NADP		NAD		NADP		NAD		NADP		NAD	
	Km (mM)	*V* (%)	*Km* (mM)	*V* (%)	*Km* (mM)	*V* (%)	*Km* (mM)	*V* (%)	*Km* (mM)	*V* (%)	*Km* (mM)	*V* (%)
Glucose 6-P	0.013	17	0.002	6	0.13	100	0.03	27	3.3	280	0.65	67
Galactose 6-P	0.029	17	0.009	6	0.58	54	0.18	21				
Deoxyglucose 6-P	1.1	60	0.37	40	10	29	5.6	67				
Glucose	5000	313	1700	148	*		2500	250				
NADP (glucose 6-P)	0.004				0.003							
NAD (glucose 6-P)			0.001				0.003					

* Too high to measure.

Table 4. Substrate specificity of cytosol glucose 6-P dehydrogenase from rat liver. Assays were performed with the purified enzyme at pH 7.5 and 10.0 in the presence of NADP. Substrate concentrations were ranged above and below the *Km* values. *V* is given as percentage of activity with NADP and glucose 6-P at pH 10.0.

Substrate	pH 7.5		pH 10.0	
	Km (mM)	*V* (%)	*Km* (mM)	*V* (%)
Glucose 6-P	0.032	55	0.4	100
Galactose 6-P	5.9	41	100	40
Deoxyglucose 6-P	0.91	7	21	17
NADP (glucose 6-P)	0.006		0.012	

No activity was detected on glucose with either NAD or NADP as coenzyme, and also on NAD with the four substrates tested.

for NAD with any of the four substrates used, irrespective of pH, and 10 times greater at pH 9.6–10.0 than at pH 7.1–7.5. The *Km* values for glucose 6-P and galactose 6-P with NADP as coenzyme were both in an order of 10^{-5}M at pH 7.5, and that for NADP was in an order of 10^{-6}M when the substrate was glucose 6-P.

4) The *Km* was about 10^5 times greater for glucose than for glucose 6-P or galactose 6-P.

On the other hand, the *Km* values of the cytosol enzyme of rat liver estimated in the present study accorded well with previous data (Glock and McLean 1953, Kimura and Yamashita 1972, Matsuda and Yugari 1967). The enzyme closely resembled the human erythrocyte glucose 6-P dehydrogenase in that the *Km* values for glucose 6-P, galactose 6-P and deoxyglucose 6-P with NADP as coenzyme and that for NADP with glucose 6-P as substrate were in the orders of 10^{-5}, 10^{-3}, 10^{-3} and 10^{-6}M, respectively, though the assay condition varied in different experiments (Marks *et al.* 1961, Kirkman 1962, Yoshida 1966, 1967).

Effects of heat and of some compounds on the enzyme activity are summarized in Tables 5 and 6, which clearly show that the microsomal enzyme was resistant, and the cytosol enzyme was relatively sensitive to heat, SH-inhibitors and dehydroepiandrosterone. The results coincided with our previous data obtained with the enzymes separated electrophoretically on polyacrylamide gels (Hori and Matsui 1967, 1968, Mochizuki and Hori 1973).

Phospholipase C had no effect on the microsomal enzyme, suggesting that this membrane-bound enzyme would not require phospholipids for activity, if it might be contained.

Mg^{2+} and Ca^{2+} were inhibitory to the microsomal enzyme, but were slightly stimulative to the cytosol enzyme at pH 7.5; inhibition of the microsomal enzyme by Mg^{2+} was 24% and 31% and that by Ca^{2+} was 30% and 39% at concentrations of 5 mM and 10 mM, respectively. On the other hand, stimulation of the cytosol enzyme by Mg^{2+} was 5% and 9% and that by Ca^{2+} was 9% and 15% at the above concentrations, respectively. Stimulation of the cytosol enzyme by Mg^{2+} and

Table 5. Effects of chemical substances on enzyme activity. Chemicals were added to reaction mixture and enzyme activity was assayed with glucose 6-P and NADP at optimal pH. Results are expressed in percentage of the untreated control. Averages of three determinations.

Chemical substance	Microsomal glucose 6-P dehydrogenase (%)	Cytosol glucose 6-P dehydrogenase (%)
p-Chloromercuribenzoate (1 mM)	81.6	41.9
N-ethylmaleimide (2 mM)	96.4	50.0
Iodoacetamide (3 mM)	100	74.9
Dithiothreitol (2 mM)	100	100
Dehydroepiandrosterone (70 μM)	100	21.5

Table 6. Effects of heat on enzyme activity. The purified enzymes were heated at 50°C for 5, 10 and 20 min, cooled and assayed with glucose 6-P and NADP at optimal pH. The results are expressed in percentage of the untreated control. Averages of three determinations.

	5 min (%)	10 min (%)	20 min (%)
Microsomal glucose 6-P dehydrogenase	76.2	61.0	35.8
Cytosol glucose 6-P dehydrogenase	36.7	26.6	22.7

Ca^{+2} was reported by Glock and McLean (1953), but the inhibition of the microsomal enzyme by bivalent cations has not been reported with mammalian species.

The molecular weight of the microsomal enzyme estimated by polyacrylamide disc electrophoresis was 191 000±2 000 (mean±S.E., n=7), while that by dodecyl sulfate-polyacrylamide gel electrophoresis was 96 000±1 000 (n=4). This means that the enzyme has a molecular weight of about 190 000, assuming that it is a homodimer.

We have previously reported (Hori and Noda 1971) that the microsomal glucose 6-P dehydrogenase of rat designated C is degraded by tryptic digestion into two enzymatically active molecular forms designated E and E_1. The molecular weights of the E and E_1 forms artificially produced from the purified enzyme were estimated by electrophoresis without dodecyl sulfate as 136 000±4 000 (n=6) and 120 000 (n=2), respectively, while that of the E form estimated by dodecyl sulfate-polyacrylamide gel electrophoresis was 66 500±1 000 (n=4). This means that the E form has a molecular weight of about 135 000, if it is of dimeric structure. For some reasons, the E_1 form did not show a protein band on dodecyl sulfate-polyacryl-amide gels, though it did on dodecyl sulfate-free gels.

The above data indicate that the change of the microsomal enzyme from the C form into E_1 form is the result of tryptic removal of not only minor fragments of polypeptides as previously predicted (Hori and Noda 1971) but rather large fragments amounting to 70 000 in molecular weight.

The molecular weight of 190 000 of the microsomal enzyme reported here is considerably different from that of rat and deer mouse hexose 6-P dehydrogenase (135 000) reported by Shaw and Koen (1968), but it is rather close to the value of human hexose 6-P dehydrogenase (223 000) reported by Srivastava *et al.* (1972).

In this connection, it is worth mentioning our unpublished finding that degradation of the microsomal enzyme by trypsin or tissue proteinases into enzymatically active smaller molecules is a phenomenon common to all the vertebrate enzymes studied, which include fish, frog, snake, chicken and rodent enzymes.

The molecular weights of two major forms of the cytosol enzyme estimated by the method of Zwaan (1967) were 110 000±4 000 (n=4) and 220 000±5 000 (n=4), being in good agreement with previous data on rat liver and human erythrocyte glucose 6-P dehydrogenases (Matsuda and Yugari 1967, Hori and Yonezawa 1972, Watanabe and Taketa 1972, Kirkman and Hendrickson 1962, Tsutsui and Marks 1962).

Summary

The microsomal glucose 6-phosphate dehydrogenase has been purified about 3 300-fold from rat liver by CM- and DEAE-Sephadex column chromatography. It has a molecular weight of 190 000 and has optimal activity at pH 10.8. It is insensitive to SH-inhibitors, dehydroepiandrosterone and heat, but is inhibited by Mg^{2+} and Ca^{2+} (5–10 mM). The *Km* values for glucose 6-P, galactose 6-P and deoxyglucose 6-P with NADP as coenzyme and those for NAD and NADP with glucose 6-P as substrate at pH 7.5 were 13, 29, 1 100, 4 and 1 μM, respectively. Such properties were in striking contrast to the properties of the cytosol glucose 6-P dehydrogenase (D-glucose 6-phosphate:NADP oxidoreductase, EC 1.1.1.49) partially purified from rat liver (122 units/mg protein), but closely resembled the properties of the hexose 6-P dehydrogenase of mouse liver microsomes reported by Beutler and Morrison (1967, *J. Biol. Chem.* 242, 5289–5293).

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