The Latency of Hexose 6-phosphate Dehydrogenase Activity in Rainbow Trout Liver Microsomes

By

Takayuki Takahashi, Koh-ichi Oka
and **Samuel H. Hori**

Zoological Institute, Hokkaido University

(With 5 Text-figures and 6 Tables)

Hexose 6-phosphate dehydrogenase (H6PD) is a microsomal enzyme which seems to be functioning *in vivo* as a glucose 6-phosphate dehydrogenase (Srivastava and Beutler, 1969). The enzyme may possibly be involved in the microsomal electron transport systems (Mandula *et al.*, 1970; Hori and Takahashi, 1974), but further studies are still required for establishing this concept.

We have previously reported that rat liver microsomal H6PD is a latent enzyme, like UDP-glucuronyltransferase (Vessey and Zakim, 1971; Winsnes, 1969; Graham and Wood, 1974), glucose 6-phosphatase (Nordlie and Arion, 1964; Stetten and Burnett, 1966; Nilsson *et al.*, 1973), and nucleoside diphosphatase (Nilsson *et al.*, 1973; Ernster and Jones, 1962; Kuriyama, 1972), and that the latency is primarily due to the inaccessibility of NADP to the enzyme which is located on the luminal side of the microsomal membranes (Hori and Takahashi, 1977; Takahashi and Hori, 1978). It appears thus likely that the activity of H6PD might be regulated by the physical state of membrane phospholipids.

The purpose of the present study is to examine whether the latency of H6PD activity is a phenomenon common to all vertebrate species or restricted only to the rat and whether the cause of the latency would be ascribed to the impermeability of microsomal membranes to NADP in rainbow trouts as in rats. The reason why rainbow trouts were chosen as materials is that the H6PD activity is high, but its latency is low in this species when compared with other vertebrates examined. Such properties of rainbow trout H6PD facilitate the kinetic studies of H6PD on intact microsomes.

Materials and Methods

Animals and reagents

Animals used were shown in Table 1. Phospholipase A from *Crotalus durissus*

terrificus venom was purchased from Sigma; Ultrogel AcA 34 from LKB and protein assay kit from Bio-Rad Laboratories. Emulgen 913 was a gift of Kao-Atlas Co.. The sources of other reagents used were listed in previous reports (Hori and Takahashi, 1977; Takahashi and Hori, 1978).

Preparation of microsomes

Livers were homogenized in 9 volumes of 0.25 M sucrose, and the microsomes were isolated by centrifuging the 12,000 × g supernatant at 144,000 × g for 60 min. The microsomal pellet was suspended in 0.25 M sucrose (2 volumes of original tissues) and kept at 2°C.

Enzyme assays

H6PD activity was assayed spectrophotometrically. The assay mixture contained in 1.0 ml: 50 mM Tris-HCl buffer (pH 7.5)/0.24 mM NADP/3.6 mM deoxyglucose 6-phosphate or glucose 6-phosphate or galactose 6-phosphate and enzyme. Reaction was started by the addition of hexose 6-phosphate and extinction changes at 340 nm were measured for 5 min. Unless otherwise stated, enzyme assay was done at 20°C. One unit of activity is defined as the amount of enzyme required to produce one μmol of NADPH per min. Latency of microsomal H6PD activity was expressed as: [1-(activity in intact microsomes/activity in detergent-treated microsomes)]×100.

Glucose 6-phosphatase activity was assayed at 20°C as described previously (Takahashi and Hori, 1978).

NADPH-cytochrome c reductase activity was measured at 20°C using 50 mM Tris-HCl buffer (pH 7.5)/0.30 mM NADPH/0.05 mM cytochrome c/1 mM KCN in 1.0 ml. The reduction of cytochrome c was followed at 550 nm.

Protein concentration was determined with a Bio-Rad protein assay kit (Bradford, 1976), using bovine serum albumin as a standard.

Partial purification of rainbow trout liver H6PD

Microsomal pellets were suspended in 3 volumes of 20 mM phosphate buffer (pH 6.8) containing 0.1 M KCl and 1 mM EDTA, and sonicated for 5 min at 2°C. The sonicated microsomes were centrifuged at 144,000 × g for 40 min, and the resulting supernatant was subjected to gel filtration on Ultrogel AcA 34 which was equilibrated with 20 mM phosphate buffer (pH 6.8) containing 0.1 M KCl and 1 mM EDTA. The flow rate was 30 ml per hr and fractions of 5 ml were collected. The fractions containing H6PD activity were pooled.

Results and Discussion

Latency of H6PD in vertebrate liver microsomes

Table 1 shows the H6PD activity of liver microsomes isolated from various animals. As is evident in this table, the enzyme exists in a latent form without

exception, though the degree of latency varies in different species. It is of particular interest that fishes exhibit a high level of enzyme activity, but the degree of latency is low when compared with mammals, reptiles and amphibians. Since such properties of fish enzymes are of great advantage to the study of enzyme kinetics in intact microsomes, we used rainbow trouts in the following experiments.

Table 1. Latency of H6PD activity in vertebrate liver microsomes. Enzyme activities of intact microsomes and of Triton X-100-treated microsomes were assayed with NADP and deoxyglucose 6-phosphate as substrates. Assay temperature was 37°C, except that the rainbow trout enzyme was assayed at 20°C.

Species (No. of animals)		Enzyme activity (*m*units/mg protein) of microsomes after treatment with		Latency
		None	Triton X-100 (0.5%, 30 min, 20°C)	
Mammalia				
Rat	*Rattus norvegicus* (5)	1.06	21.34	95.0
Mouse	*Mus musculus* (3)	1.70	20.15	91.6
Hamster	*Mesocricetus auratus* (2)	0.83	16.70	95.0
Rabbit	*Oryctolagus cuniculus* (2)	0.93	18.06	94.9
Aves				
Domestic fowl	*Gallus domesticus* (1)	1.22	2.14	43.0
Reptilia				
Snake	*Elaphe quadrivirgata* (3)	0	6.79	100
Amphibia				
Frog	*Rana chensinensis* (2)	0.51	14.03	96.4
Clawed toad	*Xenopus laevis* (3)	0	3.14	100
Pisces				
Carp	*Cyprinus carpio* (3)	10.28	69.76	85.3
Crucian carp	*Carassius carassius* (6)	14.41	81 64	82.3
Rainbow trout	*Salmo gairdnerii irideus* (8)	13.32	38.24	65.2

Organ specific distribution of H6PD in rainbow trout

The specific activity of microsomal H6PD in various organs of rainbow trout is listed in Table 2. The activity was outstandingly high in the liver, while the heart and skeletal muscle showed no detectable level of enzyme activity even in the presence of Triton X-100. The latency of H6PD activity was remarkable in the liver and intestine.

Activation of H6PD by detergents, lipid hydrolases and sonication

The specific activity of microsomal H6PD in rainbow trout livers ranged from 16.5 to 76.6 *m*units/mg protein in different individuals, but the latency of activity was relatively constant (61.9±3.2, n=8). The mean activities and standard errors (n=8) of intact and detergent-treated microsomes were 13.3±2.1

and 38.2±9.0 *m*units/mg protein, respectively. Figs. 1 and 2 show the effects of various treatments on the latency of H6PD activity. The enzyme was activated to the same extent by 0.2% deoxycholate, 0.4–0.5% Triton X–100 and 0.3–0.5%

Table 2. H6PD activity in the microsomal fractions from various organs of rainbow trout. Enzyme activities of intact microsomes and of Triton X–100-treated microsomes were assayed with NADP and deoxyglucose 6–phosphate.

Organs	Enzyme activity (*m*units/mg protein) of microsomes after treatment with		Latency
	None	Triton X–100 (0.5%, 30 min at 20°C)	
Liver	14.9	33.1	55.0
Spleen	2.48	2.51	1.2
Kidney	2.84	2.89	1.7
Testis	2.60	3.10	16.1
Intestine	0	2.90	100
Brain	2.24	2.56	12.5

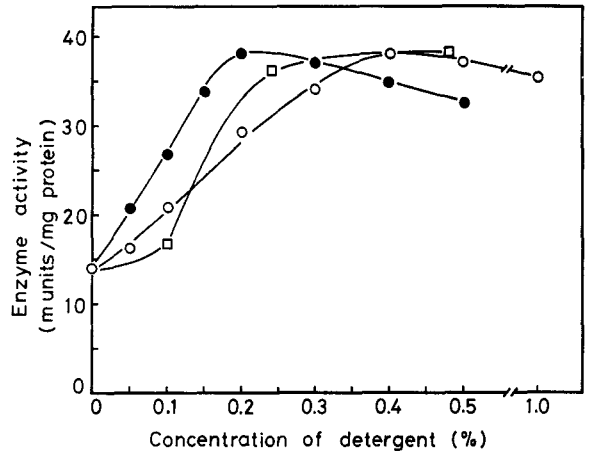

Fig. 1. Effects of detergents on microsomal H6PD activity. Freshly prepared microsomes (11.7 mg microsomal protein/ml) were treated with Triton X–100 (○–○) or Emulgen 913 (□–□) or Na deoxycholate (●–●) for 10 min at 20°C, and then the activity was assayed with deoxyglucose 6-phosphate and NADP.

Emulgen 913 within 10 min after treatment (Fig. 1), and the activated enzyme was all stable at 20°C at least for 3 hours.

The extent of activation by lipid hydrolases was dependent on the time of treatment and on the concentration of the hydrolases used (Fig. 2). When

compared with the previous data on rat H6PD (Hori and Takahashi, 1977), it is evident that the rainbow trout enzyme differs from the rat enzyme in the following respects: (1) Cholesterol esterase activates 70% of the rat enzyme, but it has little effect on the rainbow trout enzyme, and (2) phospholipase C treatment causes about 90% activation of the rainbow trout enzyme, while it results in only 50% activation of the rat enzyme. Such differences might be due to the difference in the lipid composition of microsomal membranes between the two species.

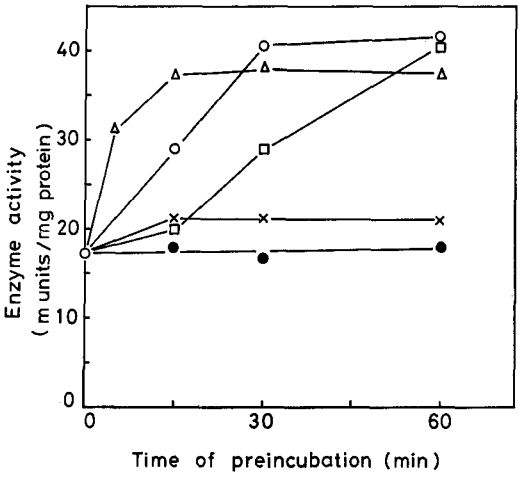

Fig. 2. Effects of lipid hydrolases on microsomal H6PD activity. Freshly prepared microsomes (9.3 mg protein/ml) were incubated with phospholipase A (□–□, 1.4 μg/mg microsomal protein) or phospholipase C (△–△, 157 μg/mg microsomal protein) or pancreatic lipase (○–○, 252 μg/mg microsomal protein) or with cholesterol esterase (×–×, 755 μg/mg microsomal protein) at 20°C in the presence of 12.5 mM Tris-HCl buffer (pH 7.5) containing 2 mM $CaCl_2$. The hydrolases were replaced by the Tris buffer containing $CaCl_2$ for control (●–●). H6PD activity was assayed with deoxyglucose 6-phosphate.

The activation by Emulgen 913 and sonication seemed to be solely due to solubilization (Table 3), while treatments with Triton X-100 and deoxycholate caused an incomplete solubilization. Such effect of deoxycholate is in striking contrast to the previous finding that this detergent causes 100% solubilization of the rat enzyme. The difference may again be explained in terms of the difference in microsomal lipid composition between the two species.

Properties of solubilized and membrane-bound enzymes

The Km values were determined with solubilized and membrane-bound H6PD and the results are shown in Table 4. The values did not differ significantly in the solubilized and membrane-bound forms.

Table 3. Solubilization of rainbow trout liver H6PD by various treatments. Freshly prepared microsomal suspensions were treated with Triton X–100 (0.5%, w/v), deoxycholate (0.25%, w/v) or Emulgen 913 (0.5%, w/v), or with phospholipase A (1.9 μg/mg microsomal protein), phospholipase C (150 μg/mg microsomal protein) or pancreatic lipase (343 μg/mg microsomal protein) in the presence of 12.5 mM Tris–HCl buffer (pH 7.5) containing 2 mM $CaCl_2$ for 30 min at 20°C, or sonicated for 2 min at 2°C. The treated microsomes were then centrifuged at 144,000×g for 40 min, and the supernatants were assayed for H6PD activity toward deoxyglucose 6–phosphate.

Treatments	Enzyme activity (*m*units/mg protein)	Enzyme solubilized (% of uncentrifuged)
None	18.2	0
Triton X–100	67.0	68
Na deoxycholate	67.0	75
Emulgen 913	67.0	100
Phospholipase A	67.0	9
Phospholipase C	60.3	0
Pancreatic lipase	67.0	7
Sonication	47.7	97

Table 4. Km values of solubilized, membrane-bound and partially purified H6PD. Treatments of microsomes were performed as described in legend to Table 3. Km was determined using initial reaction rates by the method of Lineweaver and Burk (1934), with 3.6 mM deoxyglucose 6–phosphate and 0.24 mM NADP.

Treatments	Deoxyglucose 6–phosphate (mM)	NADP (mM)
Intact microsomes	0.63	0.033
Sonicated, 144,000×g supernatant	0.77	0.038
Phospholipase A	0.74	0.040
Phospholipase C	0.87	0.059
Partially purified enzyme	0.83	0.037

The effect of temperature on H6PD activity is shown in Fig. 3. The Arrhenius plots of the activities of intact and Triton X–100-treated microsomes showed a straight line in the temperature range of 11° to 35°C, and the values of activation energy were substantially the same in the two microsomes, being 13.8 and 12.8 Kcal/mol, respectively. A similar value (12.5 Kcal/mol) was also obtained with detergent-treated microsomes of rat liver. This suggests that the binding to the microsomal membranes would not alter the catalytic properties of H6PD.

Another series of experiments also indicate that phospholipids do not exert influence on the enzyme activity; i.e., no change in the activity was observed after treatment of partially purified enzyme with phospholipases A and C, and with phosphatidylcholine and phosphatidylethanolamine.

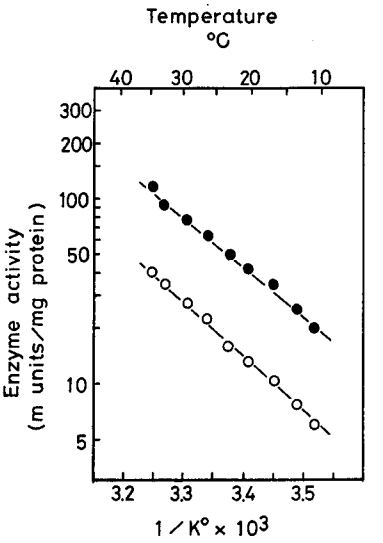

Fig. 3. Arrhenius plots of microsomal H6PD activity. H6PD activity was assayed at various temperatures with 3.6 mM deoxyglucose 6-phosphate and 0.24 mM NADP using intact microsomes (○–○) and the microsomes treated with 0.5% Triton X–100 (●–●). Rainbow trout H6PD was instable above 37°C.

Intramicrosomal localization of H6PD

Fig. 4 illustrates neutralization curves obtained when increasing amounts of rabbit anti-crucian carp H6PD antibody (supplied by Messrs. Y. Sado and N. Matsuoka) were added to a given amount of partially purified H6PD from rainbow trout liver. As is evident in this figure, the activity toward deoxyglucose 6-phosphate was most prominently inhibited by antibody. Consequently, the effect of antibody on the membrane-bound H6PD was examined using deoxyglucose 6-phosphate and NADP as substrates.

Antibody inhibited the H6PD activity of phospholipase A-treated microsomes efficiently, but it had no effect on that of intact microsomes (Fig. 5). This indicates that the antigenic site of H6PD is not exposed on the cytoplasmic side of the membranes, and that the disturbance of membrane phospholipids facilitates the antibody penetration into the membranes.

The enzyme of detergent-treated microsomes responded to antibody less markedly than that of phospholipase A-treated microsomes (Fig. 5). Taking into account that two thirds of the enzyme are solubilized by Triton X–100 treatment, but none by phospholipase A (Table 3), the above findings appear to indicate that the membranes become more permeable to antibody after the lipase treatment than after the detergent treatment.

It has been reported by DePierre and Dallner (1975) that when used at a low concentration deoxycholate is an useful detergent for introducing trypsin into the lumen of microsomal vesicles without disturbing the membrane architecture drastically. Use of trypsin in the presence of a small amount of deoxycholate thus enable us to discriminate the enzymes located on the cytoplasmic surface from

those located on the luminal surface. Table 5 shows the effects of trypsin and deoxycholate on the rainbow trout H6PD. As is clear in this table, trypsin alone has little effect on the enzyme activity, but it markedly inhibits the activity in the presence of deoxycholate. It was further demonstrated by electrophoresis that the trypsin treatment in the presence of deoxycholate results in the degradation of H6PD into enzymatically active, smaller molecular forms, as previously observed with rat microsomes.

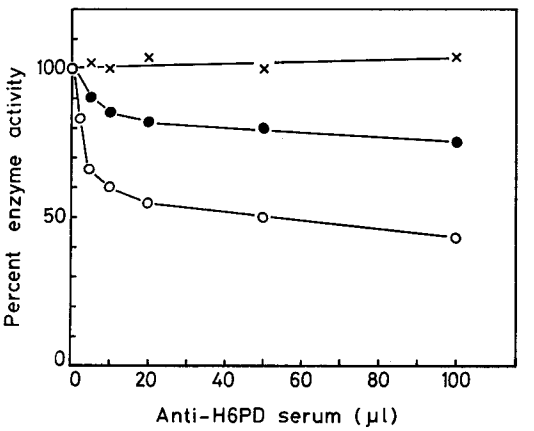

Fig. 4. Effect of antibody on the activity of partially purified H6PD. Partially purified enzyme preparations from rainbow trout liver were incubated with rabbit anti-crucian carp H6PD antiserum for 20 min at 20°C in 50 mM Tris-HCl (pH 7.5), and then the enzyme activity was assayed by the addition of NADP and one of the substrates. The substrates were glucose 6-phosphate (×–×), deoxyglucose 6-phosphate (○–○) and galactose 6-phosphate (●–●). The activities toward glucose 6-phosphate, deoxyglucose 6-phosphate and galactose 6-phosphate in the absence of antibody were 3.9, 5.6 and 6.4 *m*units, respectively. These activities were not affected by control serum.

In the case of glucose 6-phosphatase, trypsin inhibited about 40% of the total activity and the inhibition was prominently enhanced by the addition of deoxycholate.

In contrast to the two enzymes, NADPH-cytochrome c reductase was 90% inhibited by trypsin alone. These findings suggest that H6PD and glucose 6-phosphatase are located on the luminal surface and NADPH-cytochrome c reductase on the cytoplasmic surface of the membranes, just as in rat microsomes.

It remains to be seen whether the 40% inhibition of glucose 6-phosphatase by trypsin alone might be due to the penetration of trypsin into the microsomal membranes.

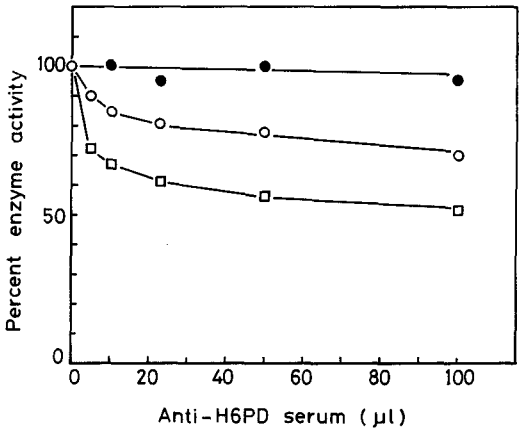

Fig. 5. Effect of antibody on microsomal H6PD activity. Microsomes were isolated from rainbow trout liver, and were treated as described in legend to Table 3. Intact microsomes (●–●), Triton X-100-treated microsomes (○–○) and phospholipase A-treated microsomes (□–□) were incubated with rabbit anti-crucian carp H6PD antiserum for 20 min at 20°C in 50 mM Tris-HCl (pH 7.5), and then the H6PD activity was assayed with NADP and deoxyglucose 6-phosphate. Intact, Triton X-100-treated and phospholipase-A-treated microsomes showed a specific activity of 16.2, 42.6 and 43.2 *m*units/mg protein, respectively.

Table 5. Effects of deoxycholate and/or trypsin on microsomal enzymes. 0.45 ml of microsomal suspensions (5.9 mg protein) were mixed with 2.5 ml of 50 mM Tris-HCl buffer/50 mM KCl/0.25M sucrose/0.041% Na deoxycholate, pH 7.5. The mixtures were incubated with or without trypsin (42.4 μg/mg microsomal protein) for 17 hr at 2°C, and centrifuged at 144,000×g for 40 min. The pellets were suspended in a small volume of 0.25M sucrose and assayed for enzyme activities. H6PD activity toward deoxyglucose 6-phosphate was measured with the microsomes treated with 0.5% Triton X-100, and glucose 6-phosphatase activity was assayed in the presence of 0.05% Triton X-100.

	Activities sedimented at 144,000×g after treatments with			
	None	Deoxycholate	Trypsin	Deoxycholate +trypsin
H6PD (*m*units)	237	144	220	51
Glucose 6-phosphatase (*n*mol Pi released/min)	189	124	107	5
NADPH-cytochrome c reductase (*n*mol cytochrome c reduced/min)	216	173	22	5

Membrane permeability to substrates

H6PD and glucose 6-phosphatase are both located on the luminal surface of microsomal membranes and are both capable of utilizing hexose 6-phosphates as substrates. It is therefore reasonable to assume that comparison of the latency of these enzymes gives some information on the permeability of the membranes to substrates (Takahashi and Hori, 1978). As shown in Table 6, the latency of glucose 6-phosphatase activity was always lower than that of H6PD. The difference is particularly evident when the enzymes are assayed with glucose 6-phosphate as substrate. This suggests that the latency of H6PD is not due primarily to the impermeability of membranes to glucose 6-phosphate, but rather to the impermeability of the membranes to NADP, the phenomenon being similar to that observed with rat microsomes.

Table 6. Comparison of the latency of H6PD and glucose 6-phosphatase activities in rainbow trout liver microsomes. Microsomes were washed once with 0.25M sucrose so as to minimize the contamination of cytosol glucose 6-phosphate dehydrogenase. Total activity of H6PD was assayed with the microsomes treated with 0.5% Triton X-100. Total glucose 6-phosphatase activity was assayed in the presence of 0.05% Triton X-100.

	None	Triton X-100	Latency
H6PD (*m*units/mg protein)			
Glucose 6-phosphate	8.8	27.8	68.3
Deoxyglucose 6-phosphate	11.0	41.7	73.6
Galactose 6-phosphate	15.5	51.0	69.8
Glucose 6-phosphatase (*n*mol Pi released/min/mg protein)			
Glucose 6-phosphate	46.8	62.3	24.9
Deoxyglucose 6-phosphate	23.4	66.3	64.7
Galactose 6-phosphate	15.0	32.7	54.1

On the basis of the above findings it may be possible to conclude that the rainbow trout H6PD is located on the luminal surface of microsomal membranes, that the membrane lipids have no direct effect on the H6PD activity, but they simply serve as a barrier which makes the enzyme inaccessible to the substrate, particularly, NADP, that this membrane impermeability to substrates may be the primary cause of the latency of H6PD activity, and that there is no fundamental difference between rainbow trouts and rats in these respects. The only species differences observed (difference in the effects of detergents and lipid hydrolases on enzyme activation and solubilization) might probably be pertinent to the difference in lipid composition of microsomal membranes between the two species.

Summary

The latency of hexose 6-phosphate dehydrogenase activity was consistently observed in liver microsomes prepared from 11 species representing 5 vertebrate orders.

Intact microsomes isolated from rainbow trout liver showed only about 30% of the total hexose 6-phosphate dehydrogenase activity. The enzyme was activated by Triton X–100, Emulgen 913, Na deoxycholate, phospholipases A and C, pancreatic lipase, and also by sonication.

The catalytic properties were the same in the solubilized and membrane-bound enzymes.

The results of the experiments with antibody and trypsin suggest that the enzyme of rainbow trout liver is bound to the inner surface of microsomal membranes. It is also suggested that the membrane impermeability to NADP is the primary cause of the hexose 6-phosphate dehydrogenase latency.

The present results on rainbow trout were essentially the same as the results reported previously on rat.

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