Properties of Hexose 6-Phosphate Dehydrogenase from a Japanese Ray, *Raja pulchra*

By

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(With 4 Text-Figures and 3 Tables)

Introduction

In higher animals there exist two enzymes which catalize NADP-linked oxidation of glucose 6-phosphate; one is glucose 6-phosphate dehydrogenase (G6PD) and the other is the enzyme known as glucose dehydrogenase (Metzger *et al.*, 1964; Beutler and Morrison, 1967) or hexose 6-phosphate dehydrogenase (H6PD; Ohno *et al.*, 1967). Although they differ strikingly in kinetic and immunological properties (Mandula *et al.*, 1970; Srivastava *et al.*, 1972; Kimura and Yamashita, 1972; Hori *et al.*, 1975; Sado and Hori, 1978), it has been suggested that both enzymes arose from a common ancestral gene (Kamada and Hori, 1970; Stegeman and Goldberg, 1971; Srivastava *et al.*, 1972; Yamauchi and Goldberg, 1973). Furthermore, a series of comparative studies on G6PD and H6PD have demonstrated that in invertebrate species examined only certain echinoderms possess the enzyme, H6PD, while G6PD is widely distributed throughout the animal kingdom (Kamada and Hori, 1970; Mochizuki and Hori, 1973, 1976; Hori *et al.*, 1975, 1977; Matsuoka *et al.*, 1977). This strongly suggests that the time of divergence of H6PD would be near the beginning of or before echinoderm evolution. More recently, Matsuoka and Hori (1980) have provided evidence that H6PD and G6PD in echinoderms are indeed immunologically related, suggesting their divergence from a common ancestral molecule.

In spite of such progress in our knowledge about the origin of H6PD, the physiological significance of H6PD yet has to be established. Precise information about the properties of H6PDs from various animals thus seems to be important for elucidating the functional significance of H6PD. H6PDs from mammals, amphibians, teleosts and starfishes have been isolated and characterized to date, but not from other groups of vertebrates. Recently, we found that a Japanese ray, *Raja pulchra*, possesses H6PD which is apparently distinguishable from the

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ray G6PD with respect to the electrophoretic mobility and substrate specificity, although Stegeman and Yamauchi (1975) reported the absence of H6PD in Agnatha (*Myxine glutinosa* and *Petromyzon marinus*) and Chondrichthyes (*Raja erinacea*). In the present study, therefore, we attempted to characterize the ray H6PD in order to compare its properties with those of H6PD from other species.

Materials and Methods

Adult rays (*Raja pulchra*) were collected at Ishikari Bay, Hokkaido.

Glucose 6-phosphate (G6P), nicotinamide adenine dinucleotide phosphate (NADP) and nicotinamide adenine dinucleotide (NAD) were obtained from Kyowa Hakko Kogyo Co., galactose 6-phosphate (Gal6P) and 2-deoxyglucose 6-phosphate (dG6P) from Sigma Chemical Co., and Ultrogel AcA 34 from LKB Produkter AB.

Enzyme assays and examinations of the effects of $MgCl_2$, p-chloromercuribenzoate and dehydroepiandrosterone on enzyme activity were carried out by the methods previously described (Hori and Sado, 1974). One unit of activity was defined as the amount of enzyme that reduced one μmole of NADP per min. Protein was determined according to Lowry *et al.* (1951).

Preparation of ray H6PD

All procedures were performed at 4°C. Livers, 100 g, stored at −70°C, were homogenized in 9 volumes of 20 mM phosphate buffer, pH 6.4, containing 1 mM ethylenediaminetetraacetate (EDTA) and centrifuged at 10,000 ×g for 10 min. The precipitate was once washed in 20 mM phosphate buffer, pH 6.4, containing 1 mM EDTA and 0.5 M NaCl, sonicated for 10 min in this buffer, and centrifuged at 144,000 ×g for 1 hr. The supernatant obtained was treated with ammonium sulfate (40 g/100 ml) and the resulting precipitate was dissolved in 20 mM phosphate buffer, pH 6.8, containing 1 mM EDTA and 0.1 M KCl, and dialyzed against the same buffer. In order to remove contaminating G6PD, the dialyzed solution was then mixed with an excess of anti-crucian carp G6PD antibody which cross-reacted with ray G6PD (Sado and Hori, 1978), allowed to stand for 3 hr at 4°C, and subjected to gel filtration on an Ultrogel AcA 34 column (25×900 mm). Flow rate was adjusted to 30 ml/hr. Fractions of 10 ml were collected and those with high H6PD activity (NAD-linked G6P dehydrogenating activity) were pooled and concentrated by ultrafiltration. This sample was electrophoretically free of G6PD and used for kinetic and immunological studies.

Immunological studies

Preparation of anti-crucian carp H6PD antibody has been described previously (Hori *et al.*, 1977). Some teleost H6PDs were prepared in the same way as for ray H6PD, using microsomal fractions obtained by centrifuging postmitochondrial supernatants of fresh liver extracts at 105,000 ×g for 1 hr. Ouchterlony agar diffusion tests were performed essentially as described previously (Sado and Hori,

1978), and after completion of development of precipitin lines the plates were washed with 20 mM phosphate buffer, pH 6.8, containing 1 mM EDTA and 0.1 M KCl for 48 hr, and stained for enzyme activity in the medium containing 70 mM tris-Cl buffer, pH 7.5, 1.2 mM G6P, 0.2 mM NADP, 0.3 mM nitro blue tetrazolium, and 0.07 mM phenazine methosulfate.

For examining the effects of anti-crucian carp H6PD antibody on ray H6PD, the following two methods were employed: (1) Given amounts of antiserum were treated with 0 to 100 munites of enzyme. After incubation at room temperature for 3 hr and at 4°C overnight, the mixtures were centrifuged at 1,500 $\times$g for 15 min, and the activity of both the supernatant and the precipitate fractions were assayed at pH 10.0 using G6P as substrate. The amount of H6PD precipitated by 1 ml of antiserum, which was calculated from extrapolation of activity in supernatant fractions in antigen-excess regions, was referred to as precipitation potency of antibody. (2) A series of H6PD solutions, each containing 1.6 munits of activity (G6P as substrate), were mixed with increasing amounts of antiserum and diluent (0.7 ml of tris-Cl buffer, I=0.1, pH 7.5, 50 μl of 12 mM NADP, water and normal serum) in a total volume of 0.95 ml. The mixture was allowed to stand for 20 min at 21°C and the reaction was initiated by adding 50 μl of 120 mM substrate. The rate of NADP reduction was recorded spectrophotometrically at 21°C.

Results and Discussion

Figure 1 shows the pH-activity curves of ray H6PD. The enzyme utilized NAD as well as NADP as coenzyme and was active on G6P, Gal6P and dG6P especially in a high pH range. The enzyme was also active, though to a very limited extent, on glucose, like the H6PD from other species so far studied.

Table 1 shows the *Km* values of the enzyme for substrates at pH 7.5. When compared with data available on H6PDs from other species, it is clear that ray H6PD has kinetic properties similar to those of H6PDs reported previously (Table 2). Furthermore, the *Km* values of H6PDs from different sources for G6P, NADP and NAD fall within narrow ranges of 2–13 μM, 1–4 μM and 1–2.6 μM, respectively, and hence, it is likely that H6PD has not undergone marked changes with respect to the affinities for these ligands during the course of vertebrate evolution.

The NADP-linked G6P oxidation rate of ray H6PD was inhibited 5% by $MgCl_2$ and 19% by 1 mM p-chloromercuribenzoate. Inhibition by Mg^{2+} of G6P oxidation appears to be a phenomenon common to vertebrate and starfish H6PDs, while the effects of ions have been known to vary with substrates (Stegeman and Yamauchi, 1975). Dehydroepiandrosterone (70 μM) had no effect on ray H6PD.

The molecular weight of ray H6PD was estimated as approx. 230,000 by means of gel filtration on Ultrogel AcA 34 (Hori *et al.*, 1977). This is close to the values of H6PDs from rat (Hori and Sado, 1974), crucian carp (Sado and Hori, 1976), lake trout (Stegeman and Goldberg, 1971) and starfish (Matsuoka *et al.*, 1977),

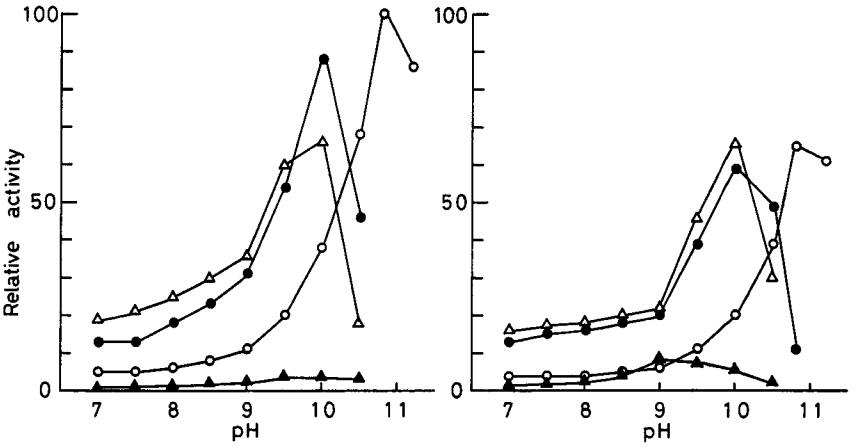

Fig. 1. Effect of pH on ray H6PD. Left, activity with NADP; right, activity with NAD. Concentration of substrate: G6P, Gal6P and dG6P, 6.0 mM; glucose, 0.2 M; NADP and NAD, 0.6 mM. Activity was expressed as percentage of the peak activity with G6P and NADP. ○—○, G6P; ●—●, Gal6P; △—△, dG6P; ▲—▲, glucose.

Table 1. Substrate specificity of ray H6PD. Assays were performed at pH 7.5 at various substrate concentrations ranging above and below *Km* values, except for glucose which did not saturate the enzyme even at 1.4 M. The *Km* values were obtained from Lineweaver-Burk plots. *V* is given as percentage of activity with G6P and NADP at pH 7.5.

Substrate	NADP		NAD	
	Km (mM)	*V* (%)	*Km* (mM)	*V* (%)
G6P	0.003	100	0.002	88
Gal6P	0.011	286	0.009	306
dG6P	0.017	423	0.011	330
Glucose	3,000	286	5,000	1,020
NADP(G6P)	0.001		0.001	
NAD (G6P)				

suggesting that the size of the H6PD would have not changed drastically during evolution.

In order to obtain further information about the properties of ray H6PD, immunological studies were carried out using antiserum against crucian carp H6PD. Although no precipitin line was observed on agar between ray H6PD and anti-crucian carp H6PD antiserum, a cross-reaction between the two became evident when the agar plate was stained for enzyme activity (Fig. 2). It was also found that the antibody had a potency to precipitate ray H6PD as well as H6PDs from

Table 2. Michaelis constants of H6PD at physiological pH.

	G6P (NADP)	Gal6P (NADP)	dG6P (NADP)	glucose (NADP)	NADP (G6P)	NAD (G6P)	Reference
	μM	μM	μM	M	μM	μM	
Mouse	5	7	66	4.065	4*	-	1)
Rat	13	29	120	5.0	4	1	2), 3)
Toad	3	140	1,300	0.32	2	1	4)
Brook trout	8.4	9.3	770**	0.0303**	2.4	2.6	5)
Crucian carp	2	9	180	0.310	2	1	4)
Ray	3	11	17	3.0	1	1	this study
Starfish	8	43	490	1.6**	1	1	6)

References: 1) Beutler and Morrison, 1967; 2) Hori and Sado, 1974; 3) Hori and Takahashi, 1977; 4) Sado and Hori, 1976; 5) Stegeman and Goldberg, 1971, 1972; 6) Matsuoka *et al.*, 1977.

* at pH 9.4

** NAD as coenzyme

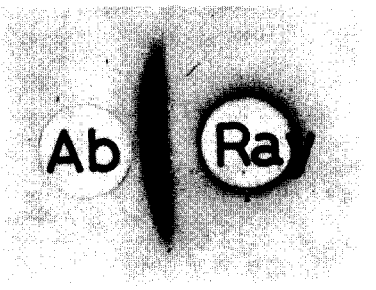

Fig. 2. Immunodiffusion plate showing a cross-reaction between ray H6PD and antiserum to crucian carp H6PD (Ab). The plate was stained for enzyme activity.

bony fishes (Fig. 3 and Table 3). The relative precipitation potency of the antibody with ray H6PD was smaller than those with H6PDs from bony fishes examined. In addition, the values appear to reflect their phyletic relationships, just as observed with teleost G6PDs and anti-crucian carp G6PD antibody (Sado and Hori, 1978).

As is evident in Fig. 4, the antibody inhibited dG6P oxidation, but rather stimulated G6P oxidation by ray H6PD, while the rate of Gal6P oxidation was not changed. Similar effects of the antibody were also observed with H6PDs from starfish (Matsuoka *et al.*, 1977) and bony fishes (unpublished). Furthermore, Takahashi and Hori (1978) have reported a multiple effect of anti-rat H6PD antibody on rat H6PD activities. Such a phenomenon that effects of antibody on the catalytic activities of H6PD vary with substrates could thus be emphasized as a general immunological properties of H6PD molecules, but it is difficult at this

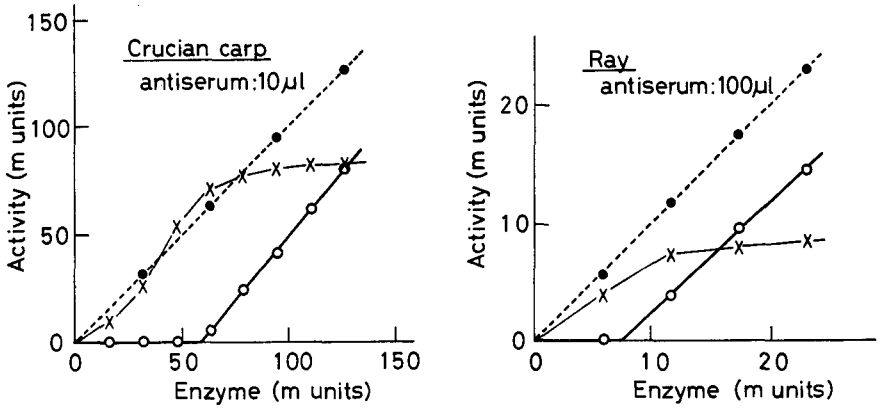

Fig. 3. Precipitation of crucian carp and ray H6PDs by anti-cruican carp antiserum. Given amounts of antiserum were treated with increasing amounts of enzyme, and centrifuged at 1,500 ×g for 15 min. o—o, activity of the supernatant fraction. ×—×, activity of the precipitate fraction. ●----●, activity without antiserum. For details, see Materials and Methods.

Table 3. Precipitation potencies of antiserum to crucian carp H6PD.

Source of H6PD	Precipitation potency (Units of H6PD precipitated by 1.0 ml of antiserum)	Ratio
Crucian carp (*Carassius carassius*)	5.9	1.00
Carp (*Cyprinus carpio*)	4.9	0.83
Cloudy-spotted loach (*Barbatula toni*)	1.73	0.29
Rainbow trout (*Salmo gairdneri irideus*)	1.65	0.28
Smelt (*Osmerus dentex*)	0.50	0.08
Rockfish (*Sebastes taczanowskii*)	0.48	0.07
Angler (*Lophius litulon*)	0.36	0.06
Cod (*Gadus macrocephalus*)	0.22	0.04
Conger eel (*Conger myriaster*)	0.48	0.08
Ray (*Raja pulchra*)	0.08	0.01

stage of knowledge to further discuss on the multiple effect of anti-crucian carp H6PD antibody on ray H6PD.

In conclusion, H6PD in a Japanese ray, *Raja pulchra*, has properties strikingly similar to those of H6PDs thus far investigated.

Summary

Hexose 6-phosphate dehydrogenase was found in a Japanese ray, *Raja pulchra*, and its kinetic and immunological properties were studied. The results indicated

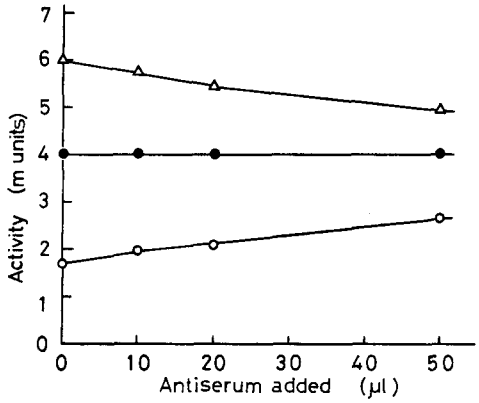

Fig. 4. Effect of anti-crucian carp H6PD antiserum on the activities of ray H6PD. Ray H6PD was incubated with increasing amounts of antiserum and the enzyme activities were assayed with NADP and G6P (o—o), Ga16P (•—•) or dG6P (△—△) as substrate. For details, see Materials and Methods.

that the ray H6PD possesses catalytic properties strikingly similar to those of H6PDs from other species and cross-reacts with antibody to crucian carp H6PD.

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