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A POSSIBLE ROLE FOR MICROSOMAL HEXOSE-6-PHOSPHATE DEHYDROGENASE IN MICROSOMAL ELECTRON TRANSPORT AND MIXED-FUNCTION OXYGENASE ACTIVITY

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SUMMARY: Reduction of cytochrome c and cytochrome P-450 was readily linked to the oxidation of glucose-6-phosphate or galactose-6-phosphate by the enzyme hexose-6-phosphate dehydrogenase in vertebrate liver microsomes. In fish liver microsomes aminopyrine demethylation and benzo[a]pyrene hydroxylation dependent on hexose-6-phosphate dehydrogenase activity was as much as 60% of that seen with an artificial NADPH generating system. Hexose-6-phosphate dehydrogenase-dependent benzo[a]pyrene hydroxylase was seen in mouse liver microsomes also, although the percent of maximal activity was less. The results are consistent with the idea that hepatic hexose-6-phosphate dehydrogenase plays a role in providing reducing equivalents for microsomal electron transport and mixed-function oxygenase reactions.

Hexose-6-phosphate dehydrogenase (H6PD) is a microsomal enzyme that oxidizes glucose-6-phosphate (G-6P), galactose-6-phosphate (Gal-6P), and other substrates including glucose[1], using either NADP or NAD as an electron acceptor (1,2). This enzyme occurs widely in animal species (3) and it is predominantly, although not exclusively, a hepatic enzyme in vertebrates such as fish and mammals, where it has been well characterized (4). H6PD has sometimes been considered as a microsomal variant of cytoplasmic glucose-6-phosphate dehydrogenase (G6PD; E.C. 1.1.1.49)(5). However, the two are genetically distinct (2,5) and the soluble G6PD functions efficiently only with glucose-6-phosphate and NADP.

The role of G6PD in the pentose phosphate pathway is well established but H6PD lacks a defined biological function. Based on subcellular distribution, there has been speculation that this enzyme may play a role in providing reducing equivalents to enzymes of the endoplasmic reticulum (6,7). In this report we now provide evidence that H6PD in vertebrates may be linked to

[1] H6PD is probably synonymous with glucose dehydrogenase, E.C. 1.1.1.47 (2).

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NADPH-dependent microsomal electron transport systems, consisting of NADPH-cytochrome c (P-450) reductase and cytochrome P-450. Specifically, we demonstrate an involvement of H6PD in reduction of cytochrome P-450, and the support mixed-function oxygenase (MFO) reactions catalyzed by this protein, in liver microsomes from fish and mammals.

MATERIALS AND METHODS: Benzo[a]pyrene (BP; Gold Label) and aminopyrine (AP) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Other substrates, including G-6P and Gal-6P, coenzymes, enzymes (G6PD) and buffers were all the best grades obtained from Sigma Chemical Co. (St. Louis, MO).

Adult marine fish were scup (*Stenotomus versicolor*), 90-310 g, caught in Woods Hole waters, and mice (*Mus musculus*) were 90 day old CD-1 females, 27-31 g, maintained as before (8). Livers were immediately dissected, pooled, and homogenized in 5 volumes of 0.25 M sucrose - 0.1 M Tris-HCl pH 7.5. Microsomal fractions were prepared as before (8) and resuspended in 50 mM Tris-HCl pH 7.8 containing 20% glycerol, 1 mM EDTA 10 mM B-mercaptoethanol, at 3 ml/g liver. Microsomes were stored in liquid nitrogen until use. H6PD activity was determined in all samples by monitoring NADPH generated (2). Electrophoretic analysis on representative samples indicated that no soluble G6PD was present in microsomal preparations.

NADPH- cytochrome c reductase activity in scup and mouse was determined as previously described (8,9). Cytochrome c reduction coupled to H6PD was determined using a reaction mixture containing 80 μM cytochrome c, about 70 ng microsomal protein per ml, and 1.9 mM G-6P or Gal-6P in 85 mM Tris-HCl pH 7.7, made to an ionic strength of 0.58 with NaCl. The reaction was initiated by adding NADP to the sample cuvette to a final concentration of 0.75 mM. Reduction rates were linear with protein.

H6PD-linked reduction of cytochrome P-450 was determined by difference spectroscopy using a Cary 118-C spectrophotometer. Both reference and sample cuvettes contained microsomes diluted to 0.7 mg protein per ml in 0.1 M Tris-HCl pH 7.5, 0.70 mM NADP, and were saturated with CO. Reduction of cytochrome P-450, initiated by adding either G-6P or Gal-6P to a concentration of 1.9 mM in the sample cuvette, was monitored by repeated scanning from 500-400 nm. Cytochrome P-450 was also determined as before (8,10).

Standard BP hydroxylase assays in 0.5 ml reaction mixtures for scup and mouse used an artificial NADPH generating system as before (8). Reaction mixtures for measuring H6PD-linked activity contained 5.2 mM NADP and either 14 mM G-6P or 13 mM Gal-6P, but no added G6PD. Reactions were initiated immediately by addition of BP, incubated at 25° for 20 minutes (scup) or 37° for 15 minutes (mouse) and hydroxylated product measured fluorometrically (11).

Standard AP demethylase was determined in 1.5 ml reaction mixtures in HEPES buffer as previously described (8). H6PD-dependent reactions were carried out using NADP and G-6P or Gal-6P as above. Reactions were started by adding recrystallized AP to 15 mM and incubated at 25° for 15 minutes, and formaldehyde was analyzed as before (12,13). Blank values for both MFO reactions were obtained by deleting the generating system, and linearity of the coupled reactions with time and protein was demonstrated with scup BP hydroxylase. Concentrations of sugar phosphates and NADP were saturating in all cases. Protein was measured according to Lowry *et al.* (14).

TABLE 1. H6PD-LINKED REDUCTION OF CYTOCHROME C AND OF CYTOCHROME P-450 IN SCUP LIVER MICROSOMES.

Cytochrome c Reductase			Cytochrome P-450		
Sample cuvette	Reference cuvette	Units/mg*	Sample cuvette	Reference cuvette	nmol/mg+
NADP	—	ND‡	CO,NADP	CO	ND
G-6P, NADP	G-6P	8.3 ± 1.6	CO,NADP, G-6P	CO,NADP	0.14 ± 0.03
Gal-6P, NADP	Gal-6P	14.1 ± 3.4	CO,NADP, Gal-6P	CO,NADP	0.13 ± 0
NADPH	—	107 ± 22**	CO, $Na_2S_2O_4$	CO	0.32 ± 0.08

‡ ND - not detectable.

*Units are nanomoles cytochrome c reduced/min/mg microsomal protein. Data represent means ± S.D. of 4-8 determinations on livers pooled from 6 fish.

+Units are nanomoles cytochrome P-450/mg microsomal protein, assuming $E_{450-490}$ = 91 mM^{-1} cm^{-1}. Data represent means ± range for replicate determinations.

**Value is typical for scup and is obtained with or without NADP and sugar phosphate also in cuvette.

RESULTS: NADPH is generated in microsomes upon oxidation of G-6P or Gal-6P by the microsomal enzyme H6PD. Reduction of cytochrome c was linked to these oxidations in scup liver microsomes. This reduction, presumably mediated by NADPH cytochrome c (P-450) reductase, showed an initial lag after which a constant rate of reduction was obtained. With either sugar phosphate this rate was only about 10% of that achieved by addition of excess NADPH *per se* (Table 1). Inhibition of H6PD by high salt may have contributed to the low reductase activity seen in the linked reaction, as the high ionic strength supporting optimal cytochrome c reduction coincidentally caused a 30-40% inhibition of H6PD activity.

The H6PD-dependent reduction of CO-bound cytochrome P-450 in scup microsomes produced a typical absorption spectrum with a Soret peak at 450 nm. The rate of cytochrome P-450 reduction that we were able to observe was not unlike the slow phase seen in rats (15), with enzymatic reduction initiated at NADPH cytochrome c (P-450) reductase rather than linked to H6PD. No further reduction was observed after 5 minutes, when the measurable content

TABLE 2. H6PD-LINKED MIXED-FUNCTION OXYGENASE ACTIVITY IN SCUP AND MOUSE LIVER MICROSOMES.

Reaction addition	Scup Liver		Mouse Liver
	Benzo[a]pyrene hydroxylase units/mg*	Aminopyrine demethylase units/mg*	Benzo[a]pyrene hydroxylase units/mg*
NADP only	14.3 ± 10.4+	46.4 ± 4.1	1.0 ± 0
NADP/G-6P	153.7 ± 2.2	86.0 ± 2.6	14.3 ± 2.0
NADP/Gal-6P	150.1 ± 1.8	75.2 ± 3.5	10.4 ± 0.6
NADP/G-6P plus G6PD	255.1 ± 5.4	132.0 ± 7.0	72.1 ± 14.0**

*Units are pmol 3-OH-BP/min or nmol HCHO/hr, per mg microsomal protein.
+Data represent means ± range for replicate assays on pooled samples.
**Mean ± standard deviation for five mice (8).

was about 40% of that obtained by reduction with dithionite (Table 1). This discrepancy in content may be related to the aerobic condition in these assays. We might expect to see additional cytochrome reduced in the linked system if oxygen were eliminated (15).

Both AP demethylase and BP hydroxylase activities in scup liver microsomes, when linked to oxidation of sugar phosphate by H6PD, proceeded at about 60% of the maximal rate obtained with added G6PD (Table 2). H6PD-dependent BP hydroxylase activity was seen in mouse liver microsomes also (Table 2). This activity was quite low, but was still 20% of that observed as maximal with the artificial NADPH generating system. Scup H6PD-dependent BP hydroxylase activity was linear with time and the rates observed were achieved without any prior incubation of microsomes with sugar phosphate and NADP. This suggests that passage of electrons from G-6P or Gal-6P to O_2, carried out by the endogenous microsomal proteins H6PD, NADPH-cytochrome c (P-450) reductase and cytochrome P-450, proceeded quite efficiently.

DISCUSSION: These results establish that the endogenous microsomal enzyme H6PD can effectively provide NADPH to microsomal electron transport systems in vertebrate liver. H6PD-dependent cytochrome reductions and MFO activities proceeded to an extent or at rates less than those seen using more artificial

means of providing NADPH. Yet, the rates seen here were great enough to argue that H6PD may play a significant role in supporting MFO activity. It remains to be shown just how closely microsomal H6PD is in fact coupled to microsomal electron transport systems in liver or other tissues where H6PD occurs. It is possible that H6PD may be an integral component functioning in microsomal electron transport.

The demonstration of a close coupling between H6PD and MFO may have important implications for studying microsomal metabolism of biological compounds such as steroids and foreign compounds such as drugs and some carcinogens. *In vitro* assays designed with MFO activity linked to H6PD might for instance provide a more realistic reflection of the function of these enzymes *in vivo*. In addition, such knowledge may further the understanding of characteristics that could be associated with variation in H6PD (16).

There is reason to speculate that MFO coupled to H6PD may confer physiological advantage. In both mammals and fish the K_m's of H6PD for both NADP and G-6P are quite low, 10^{-6} M (1, 17), while those of G6PD for the same substrates are about 10^{-5} M (18). Thus H6PD could be expected to operate more efficiently at lower concentrations of both substrates. If linked, this could conceivably alleviate dependence of microsomal electron transport functions on a number of physiological variables affecting metabolism of NADP by G6PD (19), considered by some (20) to be a major source of NADPH for MFO.

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