Ribose

AN OXIDATION PRODUCT OF GLUCOSE 6-PHOSPHATE IN MICROSMAL FRACTION*

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An oxidative metabolism of glucose 6-phosphate was studied in rat liver microsomal fraction. Although radioactive [1-14C]glucose was formed from [1-14C]glucose 6-phosphate in the microsomal fraction (Hino, Y., and Minakami, S. (1982) J. Biochem. (Tokyo) 92, 547–557), the formation was negligible when [2-14C]glucose 6-phosphate was used as a starting substrate. These results indicated an inability of the microsomal fraction to rearrange [2-14C]glucose 6-phosphate to form [1-14C]glucose 6-phosphate, and it was expected that a certain compound derived from glucose 6-phosphate accumulated as an end-product of the reaction. We, therefore, have tried to identify the product by high performance liquid chromatography, and found that ribose accumulated as the end-product. The formation of ribose was inhibited in the same manner as that of [14CO2] by antibodies against rat liver microsomal hexose-6-phosphate dehydrogenase, and the ratios of ribose to [14CO2] formed in the reaction were 0.5–0.8 on a molar basis. The finding of ribose formation further suggested the involvement of ribose phosphate isomerase and phosphatase activities in the reaction.

Hexose-6-phosphate dehydrogenase catalyzes the reduction of NAD(P)⁺ in the presence of various hexose 6-phosphates and appears to be identical with glucose dehydrogenase (β-d-glucose:NAD(P)⁺ 1-oxidoreductase, EC 1.1.1.47) (1). The activity of the enzyme is latent in microsomes, so it is necessary for maximal activity to disrupt the membrane barrier (2, 3). The physicochemical and kinetic properties of hexose-6-phosphate dehydrogenase isolated from rat liver microsomes have been reported (3, 4).

Concerning the functional aspect of this enzyme, we have previously reported that hexose-6-phosphate dehydrogenase may constitute an enzyme system together with another microsomal enzyme 6-phosphogluconate dehydrogenase (6-phospho-d-glucuronic acid:NAD⁺ 2-oxidoreductase (decarboxylating), EC 1.1.1.44) to generate reduced NAD⁺ and [14CO2] in the presence of NAD⁺ and [1-14C]G6P (5). The present work is an extension of our previous publications (3–5) and is concerned with further metabolism of G6P in the microsomal fraction. In this communication, we present results which suggest that the microsomal fraction was unable to rearrange [2-14C]G6P to form [1-14C]G6P, and that ribose accumulated as an end-product of the reaction. This is the first report to demonstrate the formation of ribose during the G6P oxidation in microsomes.

**EXPERIMENTAL PROCEDURES**

Subcellular Fractions—Microsomal and cytosol fractions were prepared as described before (3, 4), except that 5% sucrose layer was placed at the bottom of the centrifuge tube to remove glycogen particles from the microsomal fraction.

Analytical and Assay Methods—Protein concentrations were determined by the method of Lowry et al. (6) with bovine serum albumin as a reference. The reaction mixture for determining the ribose formation was essentially similar to that for the [14CO2] formation (5), it contained (final volume, 0.2 ml) 50 mM HEPES buffer (pH 7.5), 25 mM nicotinamide, 0.6% Emulgen 913, 2 mM ATP, 2 units of hexokinase, 2.5 mM NADP⁺, 10 mM glucose, and an appropriate amount of an enzyme source. When radioactive [14CO2] was to be determined, trace amount of [1-14C]glucose, [2-14C]glucose, or [6-14C]glucose was included in the reaction mixture.

For identification of the dead-end product of G6P oxidation in the microsomes, the reaction was conducted as described above using [6-14C]glucose (4 μCi), stopped by adding 5% trichloroacetic acid, and an aliquot of the clear supernatant fraction obtained by centrifugation was added with an equal volume of ethanol solution of dansyl hydrazine (10 mg/ml) (7, 8). The dansylation was carried out by incubating the mixture for 2–3 h at 30 °C. Portions of the mixtures (2 μl) were analyzed for the dansylated derivatives by HPLC (Hitachi 631A liquid chromatography), which was performed on a Merck LiChrosorb HP-5 column (particle size, 5 μm) at room temperature (20–25 °C) by increasing the concentration of acetonitrile from 5 to 30% (v/v) linearly at a flow rate of 1.0 ml/min (column pressure, 120–150 kg/cm²). The gradient was made with the solvent programmer by mixing two stock solutions of 0.2 mM KH₂PO₄ and 50% (v/v) acetonitrile (30:70, pH 7.5) and radioactivity. When measured by absorbance changes, the peak heights were made for the dansylated derivatives of phosphorylated sugars were smaller than those made by the derivatives of the corresponding nonphosphorylated sugars: e.g. the peak heights for ribose 5-phosphate and ribose 5-phosphate were about ½ and ¼ of those of ribulose and ribose, respectively. For determination of ribose formed, 1–4 μM of an authentic d-ribose was included in the incubated mixture as an internal standard.

Effects of Antibodies—The antibodies against rat liver microsomal hexose-6-phosphate dehydrogenase raised in a rabbit were shown to be specific for the antigen (3). A mixture containing microsomes, HEPES buffer (pH 7.5), Emulgen 913, and antibodies (or unimmunized globulin fraction) was preincubated for 30 min at 30 °C. For determination of the remaining activities of [14CO2] and ribose formation, ATP, hexokinase, glucose (in case of [6-14C]glucose) was included, nicotinamide, and NADP⁺ were added to make the constituents of the mixture the same as those for the assays as described above.

Chemicals—[1-14C]glucose, [2-14C]glucose, and [6-14C]glucose were purchased from Amersham and dansyl hydrizone from Tokyo Kasei (Japan). All other chemicals were of reagent grade.

**RESULTS AND DISCUSSION**

[14CO2] Was Not Formed with [2-14C]G6P as Substrate—We have determined, by measuring the formation of radioactive [14CO2], whether the microsomes have an activity to rear-
range $[2^{-14}C]G6P$ to form $[1^{-14}C]G6P$. The rationale was that, on the assumption that $[1^{-14}C]G6P$ was formed, the $1^{-14}C$ would be liberated as $^{14}CO_2$ by the actions of hexose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase present in the microsomes. The results shown in Fig. 1 indicate that, though significantly large amount of $^{14}CO_2$ was formed with $[2^{-14}C]G6P$ in the cytosol fraction, it was only negligible when the microsomal fraction was employed as an enzyme source. The molar ratios of $^{14}CO_2$ formed with $[2^{-14}C]G6P$ to that formed with $[1^{-14}C]G6P$, which could be taken as a conventional measure of the rearranging activity, were about 0.23 for the cytosol fraction and 0.01 for the microsomal fraction (determined after 10-h incubations). It could be concluded that the microsomes were unable to form $[1^{-14}C]G6P$ from $[2^{-14}C]G6P$.

Identification of Ribose—Considering the possibility that the microsomes might be deficient in the activities necessary for converting $[2^{-14}C]G6P$ to $[1^{-14}C]G6P$, we could imagine that a certain compound derived from G6P might accumulate as a dead-end product during the reaction. In order to identify the product, the reactions were conducted with $[6^{-14}C]G6P$ and the dansylated products were analyzed by the HPLC as shown in Fig. 2.

Although several absorption peaks appeared, only three major peaks (designated $a$, $b$, and $c$) could be detected when radioactivity was used for the detection. Peak $c$ decreased below the detectable level when either NADP" or microsomes was omitted from the complete system. Zero time control did not give rise to peak $c$. As shown in Fig. 3, where absorbance changes were used for the detection, a peak corresponding to peak $c$ shown in Fig. 2 increased significantly as a function of incubation time. Furthermore, peak $c$ increased in proportion to the amount of microsomal protein and the increase was inhibited by adding 2.5 mM $p$-chloromercuribenzoate or anti-hexose-6-phosphate dehydrogenase antibodies (see Fig. 5) or by heating the microsomes at 95°C for 3 min. Taken together with other control experiments (not shown), we could conclude that peak $c$ was the dansylated derivative of the dead-end product. Peak $c$ was not formed when one of the components necessary for generating G6P was omitted from the reaction mixture, so G6P formed by the hexokinase reaction might be the substrate for the reaction. Several sugars mentioned in Fig. 3 could be distinguished on the chromatogram, and we found that peak $c$ had a similar elution time with an authentic dansyl ribose. Further, the dansylated reaction mixture and the authentic dansyl ribose were combined together and the resulting mixture was subjected to HPLC, thus making it highly likely that the unknown peak $c$ corresponded to dansyl ribose. The derivatives of phosphorylated sugars were eluted at retention times between 6 and 9 min, and those of nonphosphorylated sugars between 14 and 20 min (Fig. 3). The radioactive peaks observed in the front of the chromatograms might represent polar compounds not having the dansyl group.

**Fig. 2.** Effects of removing constituents of incubation medium on the HPLC patterns. The constituents of the complete system are described under "Experimental Procedures" (1.03 mg of microsomal protein, 4 μCi of $[6^{-14}C]G6P$, NADP+, ATP, and hexokinase), and the reactions were carried out for 13.5 h at 30°C. The HPLC elution profiles of the dansylated samples were monitored by absorbance changes (broken line, arbitrary unit) and radioactivity (solid line, arbitrary unit). There appeared three major radioactive peaks designated $a$, $b$, and $c$, of which peak $c$ could be seen only in the complete system. Peaks $b$ and $c$ corresponded to the dansyl derivatives of glucose and ribose, respectively, and peak $a$, in this particular case, mostly of G6P.
The HPLC analysis of the dansylated mixtures were done as in Fig. 2, and the elution profiles were monitored by absorbance changes (arbitrary unit). The absorbance data were processed with NEC PC8001 computer after digitalization, and the profiles were drawn as a function of incubation time. Several standards and other compounds were treated with dansyl hydrazine and subjected to the HPLC under the same conditions as above, and retention times at which these compounds were eluted were determined, which these compounds were eluted were determined.

Several standards and other compounds were treated with dansyl hydrazine and subjected to the HPLC under the same conditions as above, and retention times at which these compounds were eluted were determined: 1, G6P; 2, ribose 5-phosphate, ribulose 5-phosphate, and fructose 6-phosphate; 3, erythrose 4-phosphate and xylulose 5-phosphate; 4, adenine; 5, nicotinamide; 6, glucose; 7, fructose; 8, arabinose; 9, ribose; 10, erythrose; 11, ribulose and xylulose; 12, glyceraldehyde. Several peaks eluted within 6 min were not identified precisely, but were probably derived from dansyl hydrazine and polar constituents of the reaction mixture. The chromatogram shown in the panel at the rear of the figure is the one obtained after 24-h incubation. The gradient of acetonitrile concentration (%, v/v) is also shown (-----).

ultraviolet absorption rather than radioactivity as a detection method, because relatively high background values observed in the latter method might render the results less reliable. Fig. 4 (left) shows that the height of peak c increased biphasically in the presence of NADP+, and the increase in the slow phase was similar to that observed in the control experiments carried out in the absence of NADP+. The NADP+-independent increase of peak c might not be due to the product of interest, because no appreciable radioactivity could be detected in this region when [6-14C]G6P was used as substrate and the radioactivity was employed for the detection (see Fig. 2, -NADP+). Therefore, we corrected the results by subtracting the NADP+-independent increases of the peak heights as blank from the NADP+-dependent ones (Fig. 4 (left), broken line). As shown in Fig. 4 (right), the formation of 14CO2 occurred rapidly and linearly for the initial 60 min and reached the maximum at about 2 h. On the contrary, the ribose formation was slower than the 14CO2 formation and reached the maximal level after a 6-h incubation. The maximal level of ribose formed was consistently lower than that of 14CO2 formed on a molar basis. We could estimate the molar ratios of ribose to 14CO2 accumulated at the maximum to be 0.5-0.8, though these ratios varied significantly from experiment to experiment.

Fig. 5 shows that the 14CO2 formation was inhibited in the same manner as the ribose formation by anti-hexose-6-phosphate dehydrogenase antibodies. The similarity of the inhibition patterns and the observation that ribose was formed much slower than 14CO2 might support our proposal that peak c, that was ribose, was the end product of the reaction.

Involvements of Ribose Phosphate Isomerase and Phosphatase Activities—The formation of ribose as a final reaction product suggested the involvements of ribose phosphate isomerase (D-ribose-5-phosphate ketol-isomerase, EC 5.3.1.6) and phosphatase activities in the reaction. When the microsomes were incubated with ribulose 5-phosphate, a peak corresponding to ribose appeared in addition to a ribulose peak (Fig. 6). The ribose peak was not detected, however, when ribulose was used in place of the phosphorylated compound (not shown). It could be explained as that ribulose 5-phosphate was first isomerized by the isomerase to form ribose 5-phosphate, which was then converted into ribose by a phosphatase present in the microsomes. The isomerase activity might be inhibited by p-chloromercuribenzoate, because the drug inhibited the appearance of the ribose peak, whereas the ribulose peak was less sensitive to the SH reactive drug. The contribution of cytosol enzymes to the observed activity
Ribose Formation in Microsomes

General Comments—We have presented results which indicate the formation of ribose as an end-product of G6P oxidation in the microsomes, though we are not sure whether the results can be applied to the metabolism in situ. These results are in favor of our previous report (5) which indicated that hexose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase present in the microsomal vesicles catalyzed the formation of reduced NADP⁺ through the oxidation of G6P, which was presumably transported from the cytoplasm via the G6P specific transporter (9-12). Ribose, the product of the reaction, seems to permeate the membrane barrier without any special transporting devices because of small size and having no charged groups on the molecule (13, 14), and so can leave the luminal space of the microsomal vesicles without much difficulty. No accumulation of the product within the vesicular compartment can make it possible for the enzyme system to continue to generate reduced NADP⁺ in response to demand for it.

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