THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 21, Issue of May 23, pp. 13584–13590, 1997
Printed in U.S.A.

Demonstration of a Metabolically Active Glucose-6-phosphate Pool in the Lumen of Liver Microsomal Vesicles*

(Received for publication, February 13, 1997, and in revised form, March 11, 1997)

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Glucose-6-phosphate transport was investigated in rat or human liver microsomal vesicles using rapid filtration and light-scattering methods. Upon addition of glucose-6-phosphate, rat liver microsomes accumulated the radioactive tracer, reaching a steady-state level of uptake. In this phase, the majority of the accumulated tracer was glucose, but a significant intraluminal glucose-6-phosphate pool could also be observed. The extent of the intravesicular glucose pool was proportional with glucose-6-phosphatase activity. The relative size of the intravesicular glucose-6-phosphate pool (irrespective of the concentration of the extravesicular concentration of added glucose-6-phosphate) expressed as the apparent intravesicular space of the hexose phosphate was inversely dependent on glucose-6-phosphatase activity. The increase of hydrolysis by elevating the extravesicular glucose-6-phosphate concentration or temperature resulted in lower apparent intravesicular glucose-6-phosphate spaces and, thus, in a higher transmembrane gradient of glucose-6-phosphate concentrations. In contrast, inhibition of glucose-6-phosphate hydrolysis by vanadate, inactivation of glucose-6-phosphatase by acidic pH, or genetically determined low or absent glucose-6-phosphatase activity in human hepatic microsomes of patients suffering from glycogen storage disease type 1a led to relatively high intravesicular glucose-6-phosphate levels. Glucose-6-phosphate transport investigated by light-scattering technique resulted in similar traces in control and vanadate-treated rat microsomes as well as in microsomes from human patients with glycogen storage disease type 1a. It is concluded that liver microsomes take up glucose-6-phosphate, constituting a pool directly accessible to intraluminal glucose-6-phosphatase activity. In addition, normal glucose-6-phosphate uptake can take place in the absence of the glucose-6-phosphatase enzyme protein, confirming the existence of separate transport proteins.

Liver plays a major role in regulation of blood glucose levels. In response to stress or low blood glucose levels, it releases glucose for use by other tissues. The terminal step of both glycogenolysis and gluconeogenesis, the two glucose producing pathways, is catalyzed by glucose-6-phosphatase (EC 3.1.3.9) (1). The importance of this enzyme in the regulation of blood glucose levels is clear from the debilitating effects in the absence of enzyme activity in glycogen storage disease (GSD)[1] type 1 (2). The enzyme activity was originally recovered with the microsomal subcellular fraction in the 1950s, *e.g.* de Duve *et al.* (3), which mainly derives from the endoplasmic reticulum (ER) membranes (4). The latency of its activity, discovered in early studies (5, 6), together with histochemical studies (7) indicated the compartmentation of the enzyme in the ER lumen. Consistent with this, more recent sequence information (8, 9) revealed that mammalian glucose-6-phosphatases contain the carboxyl-terminal two-lysine retention motif by which transmembrane proteins are retained in the ER by retrieval from the Golgi (10, 11). They are also very hydrophobic proteins (12), and a variety of topological studies indicate that the active site of the enzyme is located in the lumen of microsomes (13, 14).

There is no consensus of opinion to date, however, concerning the catalytic mechanism of the enzyme. Presently there are essentially two models to explain it. According to the "translocase-catalytic unit" or "substrate transport" model (15–18), the catalytic site of the glucose-6-phosphatase enzyme, situated inside the lumen of the ER (16), acts in concert with at least three putative ER transport proteins for the substrate glucose-6-phosphate and for the products phosphate and glucose, which have been named T1, T2, and T3 (2, 12, 16, 18), respectively. In the "combined conformational flexibility-substrate transport model" (19–21), there is no T1 transport protein. Instead, glucose-6-phosphatase enzyme traverses the microsomal membrane forming a water-filled space around the catalytic site in the ER membranes. The catalytic site is thus accessible from the cytosol, and the latency would be caused by the interactions between the enzyme and its membrane environment.

The substrate transport model was suggested more than two decades ago after comparing the enzyme kinetic behavior of native microsomes, which exhibit enzyme latency, and that of detergent-disrupted microsomes, *i.e.* after removal of the microsomal membrane barrier (15, 16). It was subsequently observed that various GSD 1 patients lack the enzyme activity in native but not in disrupted microsomes (2) and, most importantly, that the enzyme activities of these patients behave as predicted by the original substrate transport model (22–24). In other words, certain cases of GSD can be explained assuming the inherited deficiency of the putative transporter T1 (GSD

* This work was supported in part by an Italian Telethon grant (No. E.271) (to A. Benedetti), by grants from the Medical Research Council and Scottish Home and Health Department (to A. Burchell), by a grant from the Ciba Fellowship Trust (to A. Burchell and A. Benedetti). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of a Research Fellowship in Siena from the European Science Foundation Programme of Fellowships in Toxicology.

‖ Recipient of a Lister Institute Research Fellowship.

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[1] The abbreviations used are: GSD, glycogen storage disease; ER, endoplasmic reticulum; Mops, 4-morpholinepropanesulfonic acid.

1b), T2 (GSD 1c), or T3 (GSD 1d) (2). Liver glucose-6-phosphatase enzyme cDNAs have been cloned in humans, rats, and mice (8, 9, 25, 26), and a number of point mutations of the gene have been shown to underlie GSD 1a (27). In contrast, no mutations of this gene have been found in patients with deficiencies of microsomal glucose-6-phosphate transport (28). This indicates that the enzyme protein does not have transport function and that other loci (and proteins) are also needed for glucose-6-phosphate hydrolysis in native (intact) microsomes (2, 12, 16). Very recent evidence also indicates that the human chromosome 17 (which contains the glucose-6-phosphatase enzyme gene) is not the site of the defect in GSD 1b (29). Light-scattering transport experiments showed that liver microsomal vesicles are permeable to glucose-6-phosphate, but not to its isomers mannose-6-phosphate and glucose-1-phosphate (30), which supports a selective ER transport for the substrate of the glucose-6-phosphatase enzyme.

Despite the increasing genetic and other evidence of the existence of T1, there is still controversy. The main recent argument for the nonexistence of a T1 transport protein in the conformational model is based on the putative absence of accumulation of glucose-6-phosphate in liver microsomes incubated with the ^{14}C-labeled hexose phosphate (6, 31). In fact, it was observed that liver microsomes accumulate more ^{14}C than could be explained by the facilitative transport/passive equilibrium of added extravesicular [^{14}C]glucose-6-phosphate, and this was interpreted as a microsomal accumulation of the enzyme product ^{14}C-labeled glucose, assuming that the latter exits the microsomal lumen slowly (6, 31). In other kinetic studies (31, 32), the enzyme activity showed an initial burst phase, which was attributed to a tight coupling between glucose-6-phosphate transport and hydrolysis and thus to an extravesicular substrate pool. In addition, neither glucose-6-phosphate nor glucose uptake and accumulation have been observed in liver microsomes obtained from a GSD 1a patient (33). Very recently, knockout mice for the glucose-6-phosphatase enzyme gene have been produced (34), and it was observed that the addition of [^{14}C]glucose-6-phosphate resulted in lower uptake of ^{14}C in microsomes isolated from livers of knockout mice compared with the control, which was discussed as glucose-6-phosphatase-dependent substrate transport (34).

In contrast to this background, we have investigated the nature of the hepatic microsomal intravesicular pools deriving from the transport of glucose-6-phosphate. To this end, we have measured both [^{14}C]glucose-6-phosphate uptake and [^{14}C]glucose accumulation in rat hepatic microsomal vesicles as well as in liver microsomal preparations derived from two GSD 1a cases. Here we show that glucose-6-phosphate crosses the microsomal membrane and forms an intraluminal metabolically active pool allowing the formation of an intraluminal glucose pool whose extents were directly dependent on glucose-6-phosphatase activity. The present results confirm previous genetic evidence that the glucose-6-phosphatase enzyme is not responsible for endoplasmic reticulum glucose-6-phosphate transport and that a different T1 protein/gene is responsible for the ER transport of glucose-6-phosphate. In addition, the results provide an alternative explanation for kinetic data that were previously considered to support the conformational model.

EXPERIMENTAL PROCEDURES

Preparation of Rat Liver Microsomes—24 h-fasted male Sprague-Dawley rats (180–230 g) were used. Liver microsomes were prepared as reported (35). Microsomal fractions were resuspended in a buffer (buffer A) containing (in mM): KCl, 100; NaCl, 20; $MgCl_2$, 1; and Mops, 20, pH 7.2. The suspensions were rapidly frozen and maintained under liquid N_2 until used. Intactness of microsomal vesicles checked by measuring the latency of mannose-6-phosphatase activity (36) was greater than 90% in all the preparations employed. Microsomal protein concentrations were determined by biuret reaction using bovine serum albumin as a standard. In some experiments, microsomal glucose-6-phosphatase was inactivated by mild acidic treatment according to (37). To measure microsomal water space, microsomes were diluted (10 mg protein/ml) in buffer A containing [^{3}H]H_2O (0.2 μCi/ml) or [^{3}H(C)]inulin (0.17 μCi/ml) and centrifuged (100,000 $\times$ *g*, 60 min), and the radioactivity associated with pellets was measured to enable calculation of extravesicular and intravesicular water spaces (38, 39).

Preparation of Human Liver Microsomes—Both GSD type 1a patients were initially diagnosed by kinetic analysis of the glucose-6-phosphatase system in microsomes isolated from liver biopsy samples. Case 1 had mild symptoms until adulthood, elevated hepatic glycogen levels, abnormally low glucose-6-phosphatase enzyme activity levels (20% of age-matched control levels), and low levels of abnormally sized glucose-6-phosphatase enzyme protein as judged by immunoblot analysis.[2] Case 2 had a much more severe form of type 1a GSD with virtually all the signs and symptoms described for the disorder (2), elevated hepatic glycogen levels, virtually no glucose-6-phosphatase enzyme activity (~1% of age-matched control values), and no immunodetectable glucose-6-phosphatase enzyme protein by immunoblot analysis. Portions of liver biopsy samples from the two patients were histologically examined to check for the presence of adenoma or hepatoma cells. The need to do this is illustrated by case 2. Histology demonstrated that a first needle biopsy sample from the patient was partially adenoma and was not used for the present study (or diagnosis). In contrast, a second liver sample was found to contain only (nontransformed) liver cells and thus was used to prepare the microsomes investigated here. Human liver microsomes were prepared in 0.25 M sucrose containing 5 mM Hepes (pH 7.4) by differential centrifugation as described previously (40). The intactness of the two type 1 GSD microsomal preparations, based on the latency of *p*-nitrophenol UDP-glucuronosyltransferase activity (41), was greater than 90%. Protein concentrations were estimated by the method of Lowry as modified by Peterson (42). The study of the glucose-6-phosphatase system in human liver samples was approved by the Ethics Committee of Tayside Health Board.

Uptake Measurements—Liver microsomes (1 mg protein/ml) were incubated in buffer A containing 0.2, 0.5, 1, 5, 10, or 30 mM glucose-6-phosphate plus D-[^{14}C(U)]glucose-6-phosphate (2–3 μCi/ml) at 22 °C. At the indicated time intervals, samples (0.1 ml) were rapidly filtered through cellulose acetate/nitrate filter membranes (pore size 0.22 μm), and filters were washed with 4 ml of Hepes (20 mM) buffer (pH 7.2) containing 250 mM sucrose and 0.5 mM 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid. This latter compound was added to reduce eventual efflux of vesicular glucose-6-phosphate during the washing procedure (30). The time required to execute filtration and washing was 15–20 s. Filtration of samples of media containing no microsomes, and washing of filters as above, resulted in negligible amounts of radioactivity retained by filters. The total ^{14}C associated to microsomes retained by filters was measured by liquid scintillation counting.

Parallel filters were treated with $ZnSO_4$-$Ba(OH)_2$ to separate [^{14}C]glucose from [^{14}C]glucose-6-phosphate, and labeled glucose-6-phosphate and glucose were recovered, respectively, in the pellet and supernatant, after centrifugation (43). Briefly, washed filters were transferred into tubes containing 0.3 ml of 0.2 M $ZnSO_4$ and were pushed to the bottom. After mixing, 0.6 ml of a saturated solution of $Ba(OH)_2$ was added. Tubes were centrifuged to remove the white precipitate and filters. A 0.45-ml portion of the clear supernatant was used to measure [^{14}C]glucose produced from [^{14}C]glucose-6-phosphate by liquid scintillation spectroscopy. Routinely, the amount of [^{14}C]glucose-6-phosphate was calculated by subtracting [^{14}C]glucose from the total ^{14}C associated to microsomes measured in parallel samples (see above). In preliminary experiments, we verified i) that more than 97% of standard [^{14}C]glucose and of standard [^{14}C]glucose-6-phosphate applied on filters were recovered in the clear supernatant and in the precipitate, respectively, and ii) that direct measurements of precipitated [^{14}C]glucose-6-phosphate (after resuspending barium precipitates and filters with $ZnSO_4$-$Ba(OH)_2$ solutions and centrifuging to remove [^{14}C]glucose carry-over) gave essentially similar results.

In each experiment, alamethicin (0.05 mg/ml) was added to the parallel incubates to distinguish the intravesicular and the bound radioactivity (41, 44). The alamethicin-permeabilized microsomes were recovered on filters and washed as above. More than 95% of the microsomal protein was retained by filters, indicating that the alamethicin treatment did not affect the vesicular structure of microsomes as al-

[2] C. Hinds, and A. Burchell, manuscript in preparation.

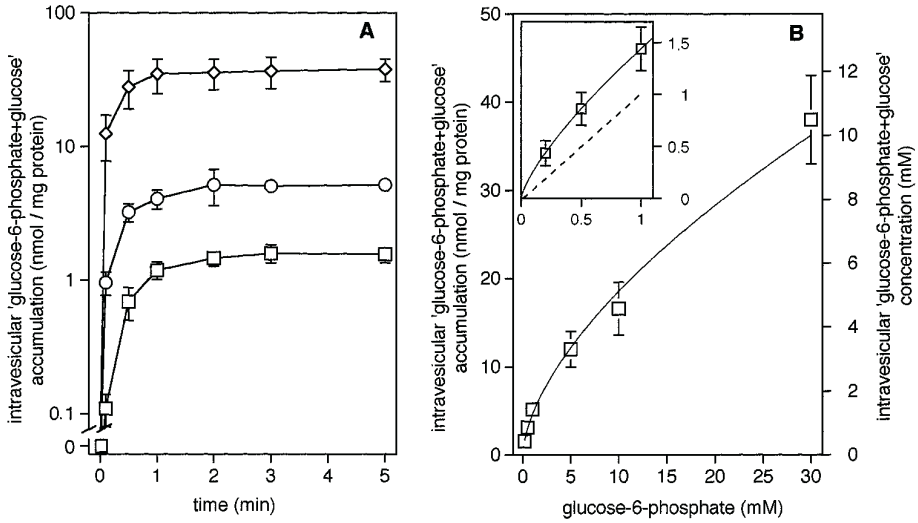

FIG. 1. **Intravesicular accumulation of glucose-6-phosphate + glucose in rat liver microsomes upon glucose-6-phosphate addition.** Microsomal vesicles (1 mg protein/ml) were incubated in the presence of various concentrations (0.2–30 mM) glucose-6-phosphate and tracer amounts of [^{14}C]glucose-6-phosphate (2–3 μCi/ml) as described under "Experimental Procedures." At indicated time points, aliquots were drawn to measure ^{14}C associated with microsomes by rapid filtration. Alamethicin (50 μg/ml) was included in parallel incubates to evaluate radioactivity unspecifically bound to microsomal membranes, and the alamethicin-releasable portion of radioactivity (regarded as intravesicular) was calculated by subtraction. Intravesicular ^{14}C was expressed as glucose-6-phosphate + glucose (see "Results"). *A*, time course of intravesicular glucose-6-phosphate + glucose accumulation upon addition of 0.2 mM (□), 1 mM (○), or 30 mM (◇) glucose-6-phosphate. Note *ordinate log scale* to better illustrate lower values. *B*, intravesicular accumulation of glucose-6-phosphate + glucose at steady-state phase (5 min of incubation) as a function of glucose-6-phosphate concentration. *Ordinate right scale* indicates intravesicular concentrations calculated on the basis of the microsomal intravesicular water content measured as described under "Experimental Procedures." The *inset* shows the enlarged initial part of the curve; the *abscissa* and the *ordinate right labels*, omitted for clarity, are those of the entire figure; concentrations of added glucose-6-phosphate are indicated by the *dotted line*. Data are means ± S.D. of three to ten experiments. *Error bars* are not visible when S.D. values were smaller than the symbol sizes.

ready reported (41). The alamethicin-permeabilized microsomes retained amounts of radioactivity ≤ 20% of that associated to untreated microsomes. Intravesicular radioactive compounds were *bona fide* lost during the washing procedure since the alamethicin nonreleasable portion did not further decrease even after extensive washing of filters (and microsomes). This allowed us to regard the alamethicin releasable portion of radioactivity as intravesicular.

To unequivocally identify the intraluminal material precipitated with barium as glucose-6-phosphate, some microsomal samples were incubated for 5 min in the presence of (30 mM) glucose-6-phosphate and in the presence or in the absence of alamethicin as described above. Samples (1 mg of protein) were filtered, and, after washing, the filters were treated with perchloric acid (3%, 1 ml). Samples were neutralized with $KHCO_3$, tubes were centrifuged to remove the precipitate and filters, and the glucose-6-phosphate content of the neutralized supernatants was measured enzymatically. To this end, 0.5 ml of the supernatant was mixed with an equal volume of buffer A containing 2 mM $NADP^+$, and NADPH formation was detected fluorimetrically (excitation and emission wavelengths were 360 and 470 nm, respectively) upon the addition of glucose-6-phosphate dehydrogenase (0.7 IU/ml). Pulse additions of standard glucose-6-phosphate (1 to 5 nmol) to the reaction mixture allowed the quantitation of microsomal glucose-6-phosphate.

Where indicated, the microsomal passive equilibration of intra- and extravesicular glucose-6-phosphate was calculated according to the formula: apparent intravesicular glucose-6-phosphate space (μl/mg of protein) = glucose-6-phosphate accumulated by microsomes (nmol/mg of protein)/concentration of added glucose-6-phosphate (nmol/μl) (30, 45).

Assay of Glucose-6-phosphatase—Glucose-6-phosphatase activity was measured after 5 min of incubation in buffer A at 22 °C on the basis of D-[^{14}C(U)]glucose production from D-[^{14}C(U)]glucose-6-phosphate according to (43). At high substrate concentrations (30 mM), the enzyme activity was also evaluated by measuring glucose production with a glucose (Trinder) kit (Sigma).

Light Scattering Measurements—Osmotically induced changes in microsomal vesicle size and shape were monitored at 400 nm at right angles to the incoming light beam using a fluorimeter (Perkin-Elmer model 650–10S) equipped with a temperature-controlled cuvette holder (22 °C) and magnetic stirrer as described elsewhere (30, 45). The mV output signals were acquired at 0.25 s intervals, using MacLab™ hardware (AD Instruments) equipped with Chart Ver. 3.2.5. software.

Materials—Glucose-6-phosphate (monosodium salt), mannose-6-phosphate (disodium salt), alamethicin, $NADP^+$, and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid were obtained from Sigma. Na_3VO_4 was from Fisher Science Co. D-[^{14}C(U)]glucose-6-phosphate (300 mCi/mMol) was from American Radiolabeled Chemicals Inc., St. Louis, MO. [^{3}H]H_2O (1 mCi/g) and [^{3}H(C)]inulin (500 mCi/g) were from DuPont NEN, Dreieich, Germany. Glucose-6-phosphate dehydrogenase (from yeast, 350 IU/ml) was from Boehringer Mannheim, Germany. Cellulose acetate/nitrate filter membranes (pore size 0.22 μm) were from Millipore. All other chemicals were of analytical grade.

RESULTS

Microsomal Uptake of Glucose-6-phosphate and Accumulation of Glucose-6-phosphate and Glucose—In a first set of experiments, rat liver microsomal vesicles were incubated in the presence of various concentrations of glucose-6-phosphate (plus [^{14}C]glucose-6-phosphate as a tracer). The radioactivity associated with microsomes was measured in vesicles incubated both in the presence and absence of the pore-forming antibiotic alamethicin (41, 44) to determine net intravesicular accumulation. Because, in addition to [^{14}C]glucose-6-phosphate, [^{14}C]glucose (produced by glucose-6-phosphatase activity) can contribute to the intravesicular measured radioactivity (as it was indeed the case, see below), we expressed as "glucose-6-phosphate + glucose" the ^{14}C-radioactivity accumulated (see Fig. 1). Glucose-6-phosphate did not likely undergo major reactions other than dephosphorylation in our incubation system, therefore glucose-6-phosphate + glucose concentrations could be calculated on the basis of the concentrations of glucose-6-phosphate added. Microsomes rapidly accumulated glucose-6-phosphate + glucose until a steady-state level was reached over a 2-min period of incubation (Fig. 1*A*). The steady-state intraluminal accumulation of glucose-6-phosphate + glucose increased by increasing the extravesicular concentration of glucose-6-phosphate although not in a linearly proportional fashion (Fig. 1*B*). Based on the measured intravesicular water space of rat liver microsomes (3.6 ± 1.1 μl/mg protein, mean ± SD, n = 6) the mM intravesicular concentrations of glucose-6-

TABLE I

Intravesicular glucose and glucose-6-phosphate content of rat liver microsomal vesicles in the steady-state phase of glucose-6-phosphate uptake

Rat liver microsomal vesicles (1 mg/ml of protein) were incubated in the presence of indicated concentrations of glucose-6-phosphate and [^{14}C]glucose-6-phosphate at 22 °C. After 5 minutes of incubation, samples were taken for the measurement of intravesicular labeled glucose-6-phosphate + glucose and glucose contents, as well as of total glucose production (*i.e.* glucose-6-phosphatase activity) as described under "Experimental Procedures." Intravesicular glucose-6-phosphate content was calculated and expressed as apparent intravesicular space as detailed under "Experimental Procedures." Results are means ± S.D. of four experiments.

	Added glucose-6-phosphate		
	0.2 mM	1 mM	30 mM
Intravesicular glucose-6-phosphate + glucose (nmol/mg of protein)	1.73 ± 0.13	5.44 ± 0.48	40.4 ± 9.9
Intravesicular glucose (nmol/mg of protein)	1.40 ± 0.10	4.88 ± 0.46	30.4 ± 9.5
Intravesicular glucose (% of total glucose formed)	4.0 ± 0.8	2.9 ± 0.5	3.2 ± 0.2
Intravesicular glucose-6-phosphate (nmol/mg of protein)	0.33 ± 0.08	0.55 ± 0.22	10.0 ± 1.6
Apparent intravesicular glucose-6-phosphate space (μl/mg of protein)	1.65 ± 0.45	0.53 ± 0.20	0.33 ± 0.05
Glucose-6-phosphatase activity (nmol/min/mg of protein)	7.4 ± 1.8	34.2 ± 5.0	192 ± 65

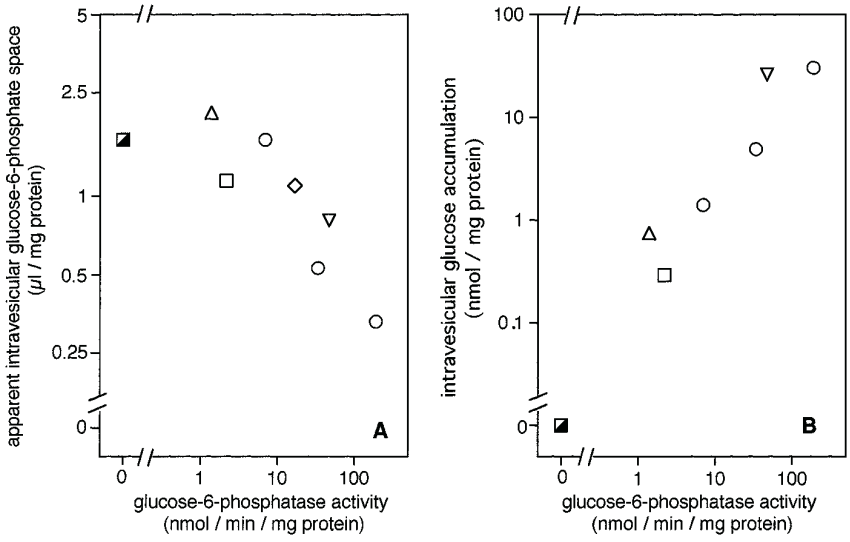

FIG. 2. **Intravesicular glucose-6-phosphate space (*A*) and intravesicular glucose accumulation (*B*) as functions of glucose-6-phosphatase activity in liver microsomes.** Microsomal vesicles were incubated as described in Table I with the following modifications: incubation temperature 37 °C (instead of 22 °C), 0.2 mM glucose-6-phosphate (◇); plus 10 μM vanadate, 30 mM glucose-6-phosphate (▽); microsomes pretreated at acidic pH to inactivate glucose-6-phosphatase, 30 mM glucose-6-phosphate (△); human microsomes from GSD 1a patients case "1", 0.2 mM glucose-6-phosphate (□); and human microsomes from GSD 1a patients case "2", 0.2 mM glucose-6-phosphate (◪). Data marked as ○ are taken from Table I. Note *log scale* for the abscissa and ordinate values to illustrate lower values. Data with rat liver microsomes are means of two to four independent experiments (S.D. of ○ is shown in Table I, other S.D. bars are not visible since they were smaller than the symbol sizes). Data with human liver microsomes are means of three measurements.

phosphate + glucose were also calculated (Fig. 1*B*). Relatively low extravesicular concentrations (≤1 mM) of glucose-6-phosphate resulted in intravesicular steady-state concentrations of glucose-6-phosphate + glucose higher than those of the extravesicularly added glucose-6-phosphate (Fig. 1*B*, *inset*; concentrations of added glucose-6-phosphate are indicated by the *dotted line*). On the other hand, at glucose-6-phosphate concentrations ≥5 mM, steady-state intravesicular concentrations of glucose-6-phosphate + glucose were lower than the added glucose-6-phosphate concentrations (Fig. 1*B*).

In theory, intravesicular concentrations of glucose-6-phosphate can maximally equal the extravesicular ones since no energy, or ion gradients, were present in the system to allow microsomal inward transport of glucose-6-phosphate over the passive equilibrium. Therefore, the higher intravesicular concentrations of glucose-6-phosphate + glucose, in the presence of glucose-6-phosphate concentrations ≤1 mM, were logically contributed to by glucose accumulation. Separate measurements of glucose and glucose-6-phosphate present in the lumen of microsomal vesicles revealed that accumulation of glucose was responsible for the apparent increase in intravesicular concentration of ^{14}C-labeled compounds over that of extravesicular glucose-6-phosphate. However, besides the intravesicular glucose pool, a glucose-6-phosphate pool could also be demonstrated by two different methods. The intravesicular steady-state glucose-6-phosphate content of the microsomes at 30 mM glucose-6-phosphate concentration was similarly estimated by either the isotopic (10.0 ± 1.6 nmol/mg protein, see Table I) or the enzymatic (11.9 ± 3.9 nmol/mg protein, mean ± SD, $n = 5$) method.

Intravesicular glucose-6-phosphate content was dependent on the extravesicular concentration of the hexose phosphate (Table I). To highlight the gradient between extra- and intravesicular glucose-6-phosphate concentrations, the apparent intravesicular glucose-6-phosphate space was also calculated. This value represents the relative intravesicular content of glucose-6-phosphate at any extravesicular concentration of the hexose phosphate investigated. Assuming a complete passive equilibrium between extra- and intravesicular glucose-6-phosphate (and no intraluminal metabolism of the hexose phosphate), this value should be equal to the intravesicular water space. The size of the measured intravesicular glucose-6-phosphate space, however, was smaller than the water space at every glucose-6-phosphate concentration investigated (Table I). Moreover, it was inversely related to glucose-6-phosphatase activity; at higher glucose-6-phosphate concentrations, which resulted in higher hydrolytic activity, the size of the apparent microsomal glucose-6-phosphate space was reduced. On the

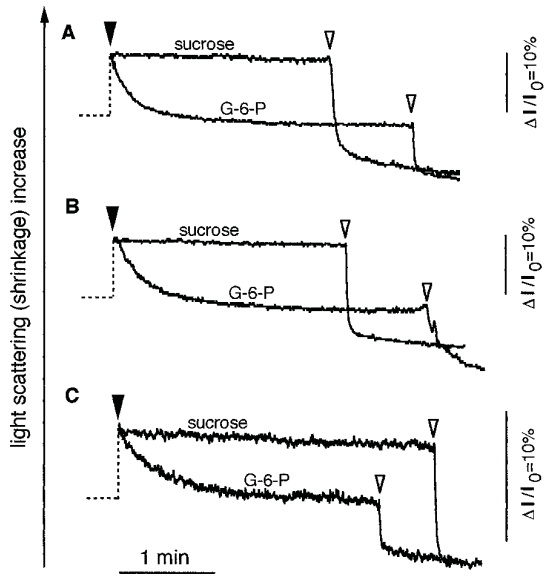

FIG. 3. **Influx of glucose-6-phosphate into liver microsomal vesicles evaluated by a light-scattering technique.** Light-scattering measurements were performed as described under "Experimental Procedures." Light-scattering increase was assumed to reflect shrinkage of microsomal vesicles (30, 45). Osmotically induced changes in microsomal vesicle size and shape were initiated by adding 0.1 ml (*black arrowhead*) of concentrate solutions of sucrose or glucose-6-phosphate (*G-6-P*) to 1.5 ml of the microsomal suspensions (in a hypoosmotic buffer; 70 or 35 μg of protein/ml, for rat and human microsomes, respectively), giving the final concentration 75 mM (sucrose) or 30 mM (glucose-6-phosphate). Alamethicin (10 μg per ml; *white arrowheads*) was then added to fully permeabilize microsomal vesicles (41). The addition of the poorly permeable sucrose resulted in a sustained shrinkage of vesicles indicating intactness of microsomal membrane (30, 45). The recovery of initial signal (swelling phase) after glucose-6-phosphate addition was assumed to reflect its entry into vesicles (30). The shrinking phase (*dotted lines*) has been graphically reconstructed by taking into account the loss of the light-scattering intensity due to dilution of microsomal suspensions after solute additions. *A*, control rat microsomes; *B*, rat microsomes plus 10 mM vanadate; *C*, human microsomes from GSD 1a patient case "2". Traces are representative of two to five separate measurements.

other hand, the absolute amount of intravesicularly accumulated glucose was directly related to the extravesicular glucose-6-phosphate concentrations and to the consecutive glucose-6-phosphatase activities, while its relative amount, expressed as the percentage of total glucose formed, was almost the same at all three glucose-6-phosphate concentrations applied (Table I).

Effect of Glucose-6-phosphatase Activity on the Intravesicular Glucose-6-phosphate Pool—Our observations suggested that the sizes of the intravesicular glucose-6-phosphate and glucose pools were governed directly by glucose-6-phosphatase activity. To prove this hypothesis, intravesicular glucose-6-phosphate and glucose contents were determined when glucose-6-phosphatase was inactivated by a mild acidic pretreatment of microsomes (16, 37), or inhibited by the competitive inhibitor vanadate (46). At low glucose-6-phosphate concentration (0.2 mM), these treatments slightly influenced the intravesicular glucose-6-phosphate levels (data not shown). At high (30 mM) glucose-6-phosphate concentration, the significant reduction of glucose-6-phosphatase activity was accompanied with an apparent increase of intraluminal glucose-6-phosphate space in both treatments (Fig. 2*A*). Similarly, microsomes of two GSD 1a patients showed very low (case 1), or virtually no (case 2), hydrolytic activity and an apparently high intraluminal glucose-6-phosphate space. In contrast, the increase of glucose-6-phosphatase activity by the elevation of the incubation temperature to 37 °C resulted in the reduction of intravesicular glucose-6-phosphate space. The apparent intravesicular glucose-6-phosphate space always showed an inverse relationship with glucose-6-phosphatase activity independently of the experimental conditions (Fig. 2*A*). The accumulation of intravesicular glucose increased as a function of glucose-6-phosphatase activity independently of the experimental conditions (Fig. 2*B*).

Effect of Glucose-6-phosphatase Activity on Microsomal Glucose-6-phosphate Transport—On the basis of the measurement of glucose-6-phosphate uptake in microsomes from glucose-6-phosphatase knockout mice, it has been suggested that the presence of the hydrolytic activity is necessary to allow efficient glucose-6-phosphate transport (34). Although the experiments above did not seem to support this suggestion, we have investigated the transport of glucose-6-phosphate in microsomes with low glucose-6-phosphatase activity (in the presence of vanadate and in samples from GSD 1a patients) using the light-scattering method (30, 45). The osmotic behavior of microsomes treated with vanadate (Fig. 3*B*) and genetically lacking glucose-6-phosphatase enzyme (human liver microsomes GSD 1a, case "2"; Fig. 3*C*) was similar to the one observed in control rat liver microsomes (Fig. 3*A*). These observations clearly indicate that microsomal glucose-6-phosphate uptake was not dependent on glucose-6-phosphatase activity. These results are also consistent with those obtained in rapid filtration experiments (Fig. 2), which further validates the use of the light-scattering technique for assaying glucose-6-phosphate microsomal transport.

DISCUSSION

Although several observations indicate the transport of glucose-6-phosphate through liver microsomal membrane (30, 47), the existence of an intraluminal glucose-6-phosphate functional pool has not been proven. Moreover, recent findings (20–21) suggest that after the addition of glucose-6-phosphate to microsomal vesicles, they accumulate mainly glucose rather than glucose-6-phosphate. It is also disputed whether glucose-6-phosphatase uses an intra- or an extravesicular substrate pool.

This study shows that liver microsomes have an intraluminal pool of glucose-6-phosphate at the steady-state phase of glucose-6-phosphate uptake although, according to previous observations (20, 21, 31), the majority of intravesicular glucose-6-phosphate has been converted to glucose. In native untreated rat liver microsomes, the size of the glucose-6-phosphate pool is directly dependent on the extravesicular glucose-6-phosphate concentration, while the apparent intravesicular glucose-6-phosphate space (*i.e.* the relative size of the pool, irrespective of the extravesicular concentrations of glucose-6-phosphate) is in an inverse relation with glucose-6-phosphatase activity (Table I). In addition, increasing glucose-6-phosphatase hydrolytic activity by elevating the temperature results in a reduction of the apparent intravesicular glucose-6-phosphate space while the decrease in enzyme activity by pH inactivation, by the competitive inhibitor vanadate and by genetic reasons, results in the highest intravesicular apparent spaces of glucose-6-phosphate. The inverse correlation between the apparent intravesicular space of glucose-6-phosphate and the enzyme activity, independently of substrate concentration, and of the treatment and source of microsomes (Fig. 2*A*) indicate that the catalytic unit directly acts on intravesicular glucose-6-phosphate.

The presence of a glucose-6-phosphatase-accessible intraluminal glucose-6-phosphate pool strongly supports the substrate transport model of the glucose-6-phosphatase system. In theory, however, these results could be explained in accordance with the modified version of the combined conformational flexibility-substrate transport model, assuming that the enzyme traverses the membrane and forms a glucose-6-phosphate

channel at the same time. The active enzyme would consume glucose-6-phosphate and release glucose into the vesicular space while, in the relative or absolute absence of hydrolytic activity, the majority of glucose-6-phosphate molecules would cross the membrane without being hydrolyzed. This possibility, however, can be excluded because of the following points. (i) Glucose-6-phosphate transport is detectable even in the absence of the enzyme protein (*i.e.* in GSD 1a case "2" in which the immunodetectable liver glucose-6-phosphatase enzyme was absent). (ii) Family studies in GSD subtype 1b (29) indicate that the genes associated with glucose-6-phosphate transport are not located on human chromosome 17, the location of the glucose-6-phosphatase enzyme gene. (iii) No mutations were found in the glucose-6-phosphatase enzyme gene in GSD 1b (28). (iv) In liver microsomes from a patient with GSD subtype 1b, glucose-6-phosphate transport could not be detected by light-scattering technique despite the presence of the enzyme.[3]

The uptake of glucose-6-phosphate into microsomes isolated from the two GSD 1a patients is consistent with uptake into microsomes from one GSD 1a patient reported previously (48), but it is in contrast to the recent report of no microsomal uptake in one GSD 1a case (33). In the latter study, the liver specimen (and microsomes) was derived from a GSD 1a patient whose liver was transplanted because of an enlarging mass. Frequently, GSD 1a patients need liver transplants because their livers contain large masses (or hepatomas), which are also surrounded by parenchyma containing multiple small foci of adenoma cells. Liver transformed cells, however, do not usually express the glucose-6-phosphatase system, and no histological analysis was presented (33), thus it is difficult to unequivocally interpret the lack of uptake reported in (33).

In microsomes from glucose-6-phosphatase knockout mice, the radioactive tracer accumulation upon the addition of glucose-6-phosphate was lower than in controls. It has, therefore, been suggested that the presence of the enzyme somehow stimulates glucose-6-phosphate transport (34). In their control mice (34), the majority of the accumulated ^{14}C was probably glucose (20, 21), and the apparently lower uptake of [^{14}C]glucose-6-phosphate in knockout mice is likely to be due to the absence of lumenal [^{14}C]glucose formation (and accumulation). In our experiments, glucose-6-phosphate transport registered by the light scattering method shows similar features in control, glucose-6-phosphatase enzyme deficient, or vanadate-treated microsomes (Fig. 3). Therefore, it is very unlikely that the glucose-6-phosphatase enzyme is, or directly cooperates with, the transporter.

The continuous consumption of the intravesicular glucose-6-phosphate pool by glucose-6-phosphatase means that the intravesicular concentration of glucose-6-phosphate must always be lower than the extravesicular one. The highest apparent glucose-6-phosphate space in native untreated rat liver microsomes was ~1.6 μl/mg of protein at 0.2 mM extravesicular glucose-6-phosphate concentration. This value is probably close to the maximal size of the glucose-6-phosphate-accessible microsomal space since similar spaces can be observed in vesicles with little or no enzyme activity, *i.e.* in the GSD type 1a human liver microsomes, and in rat liver microsomes pre-incubated at low pH (see Fig. 2). We have previously obtained comparable values for glucose-6-phosphate-accessible microsomal space by diluting vanadate-treated, [^{14}C]glucose-6-phosphate-preloaded rat liver microsomes (30). Thus, the glucose-6-phosphate-accessible space appears to constitute only a fraction of the total water space of microsomes, which can theoretically be explained by a steady-state partial equilibration of glucose-6-phosphate across the microsomal membrane or/and by uneven distribution of the glucose-6-phosphatase system in microsomal vesicles. The latter possibility is not entirely consistent, however, with cytochemical ultrastructural evidence for a widespread distribution of glucose-6-phosphatase enzyme activity within liver endoplasmic reticulum *in situ* (7). In addition, liver microsomal preparations may well contain a (small) proportion of vesicles from membranes other than endoplasmic reticulum and lacking glucose-6-phosphate transporters. Several undetermined factors may also contribute to the uncompleted equilibration of extravesicular glucose-6-phosphate with microsomal intralumenal space, *e.g.* the presence of fixed negative internal charges (45) and/or the preferential transport of one of the dissociated forms of glucose-6-phosphate (16).

A transmembrane gradient of glucose-6-phosphate is present at all extravesicular glucose-6-phosphate concentrations studied, which is consistent with the phenomenon of latency exhibited by glucose-6-phosphatase activity; the lower intravesicular concentration of glucose-6-phosphate can account for the higher K_m value of glucose-6-phosphatase in native microsomal vesicles (15). The reduction of vesicular glucose-6-phosphate space at higher extravesicular glucose-6-phosphate concentration means that the hydrolytic capacity is higher than that of the transport, in accord with the rate-limiting property of the latter. The intravesicular accumulation of glucose shows that, in the pre-steady-state phase, the velocity of glucose-6-phosphate hydrolysis surpasses that of the glucose transport. The relative accumulation of glucose (intravesicular glucose as percent of total glucose, Table I) does not increase when glucose-6-phosphatase activity and the consecutive intravesicular glucose content are increased. This finding is consistent with a low rate of glucose efflux from microsomes preloaded with glucose derived from intravesicular glucose-6-phosphate hydrolysis[4] and with the previous observation for a slow release of radioactivity (presumably mostly labeled glucose) after pre-equilibration of liver microsomes with labeled glucose-6-phosphate (20).

Collectively, our results further validate the substrate transport model of the glucose-6-phosphatase system. The existence of the intravesicular glucose-6-phosphate and glucose pools upon addition of glucose-6-phosphate, the fact that the content of both pools is governed by glucose-6-phosphatase activity, and the gradient between the extra- and intravesicular concentrations of glucose-6-phosphate are consistent with an intraluminal enzyme activity supported by transporters for substrate and products.

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