Novel Arguments in Favor of the Substrate-Transport Model of Glucose-6-Phosphatase

Isabelle Gerin, Gaëtane Noël, and Emile Van Schaftingen

The purpose of this work was to discriminate between two models for glucose-6-phosphatase: one in which the enzyme has its catalytic site oriented toward the lumen of the endoplasmic reticulum, requiring transporters for glucose-6-phosphate, inorganic phosphate (Pi), and glucose (substrate-transport model), and a second one in which the hydrolysis of glucose-6-phosphate occurs inside the membrane (conformational model). We show that microsomes preloaded with yeast phosphoglucose isomerase catalyzed the detritiation of [2-^{3}H]glucose-6-phosphate and that this reaction was inhibited by up to 90% by S3483, a compound known to inhibit glucose-6-phosphate hydrolysis in intact but not in detergent-treated microsomes. These results indicate that glucose-6-phosphate is transported to the lumen of the microsomes in an S3483-sensitive manner. Detritiation by intramicrosomal phosphoglucose isomerase was stimulated twofold by 1 mmol/l vanadate, a phosphatase inhibitor, indicating that glucose-6-phosphatase and the isomerase compete for the same intravesicular pool of glucose-6-phosphate. To investigate the site of release of Pi from glucose-6-phosphate, we incubated microsomes with Pb^{2+}, which forms an insoluble complex with Pi, preventing its rapid exit from the microsomes. Under these conditions, ~80% of the Pi that was formed after 5 min was intramicrosomal, compared with <10% in the absence of Pb^{2+}. We also show that, when incubated with glucose-6-phosphate and mannitol, glucose-6-phosphatase formed mannitol-1-phosphate and that this nonphysiological product was initially present within the microsomes before being released to the medium. These results indicate that the primary site of product release by glucose-6-phosphatase is the lumen of the endoplasmic reticulum. *Diabetes* 50:1531–1538, 2001

Since the finding that glucose-6-phosphatase, an enzyme playing a major role in glucose production during starvation, is associated with the endoplasmic reticulum (1), much work has been carried out to identify its constituents and to determine their respective role. According to the substrate-transport model (2,3), the glucose-6-phosphatase system comprises a relatively nonspecific hydrolase, the catalytic site of which is oriented toward the lumen of the endoplasmic reticulum, a specific transporter for glucose-6-phosphate and transporters for inorganic phosphate (Pi) and glucose (Fig. 1*A*). This model accounts for several kinetic observations (2–5), including the fact that glucose-6-phosphatase acts much better on glucose-6-phosphate than on mannose-6-phosphate in intact liver microsomes, whereas it is about equally active on both substrates in detergent-treated (disrupted) microsomes. Furthermore, this model allows rationalization of the effects of chlorogenic acid and some of its derivatives (such as S3483, used in this work), which inhibit the hydrolysis of glucose-6-phosphate but not of inorganic pyrophosphate in intact microsomes, having no effect in detergent-treated microsomes. These compounds are thought to be inhibitors of the glucose-6-phosphate translocase (6–8).

The substrate-transport model also accounts for the observation that two principal types of glucose-6-phosphatase deficiency have been identified: one, in which the phosphohydrotase is deficient, is known as glycogen storage disease type Ia (rev. in 9); the other, in which no transport of glucose-6-phosphate can be demonstrated in liver microsomes (10), is GSD type Ib, or GSDIb. The cDNAs mutated in GSD Ia (11,12) and GSD Ib (13) have been identified. The second encodes a protein belonging to the same family as transporters (UhpT) and a putative receptor (UhpC) for hexose-6-phosphates. Although coexpression of glucose-6-phosphatase with the protein mutated in GSD Ib in COS-1 cells reconstitutes some degree of glucose-6-phosphate transport into microsomes (14), a clear demonstration that the protein mutated in GSD Ib acts as an independent glucose-6-phosphate translocase is still missing, owing partly to difficulties in expressing the protein in heterologous systems (I.G., unpublished results).

Furthermore, measurements of the incorporation of radioactivity into liver microsomes incubated with radiolabeled glucose-6-phosphate show that only a fraction (at most ~10%) of the liberated glucose or Pi can be found inside the microsomes (15,16). This led some authors to conclude that glucose-6-phosphate transport into the en-

From the Laboratory of Physiological Chemistry, ICP and Université Catholique de Louvain, Brussels, Belgium.

Address correspondence and reprint requests to E. Van Schaftingen, UCL 7539, Avenue Hippocrate 75, B-1200 Brussels, Belgium. E-mail: vanschaftingen@bchm.ucl.ac.be.

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GSD, glycogen storage disease; Pi, inorganic phosphate.

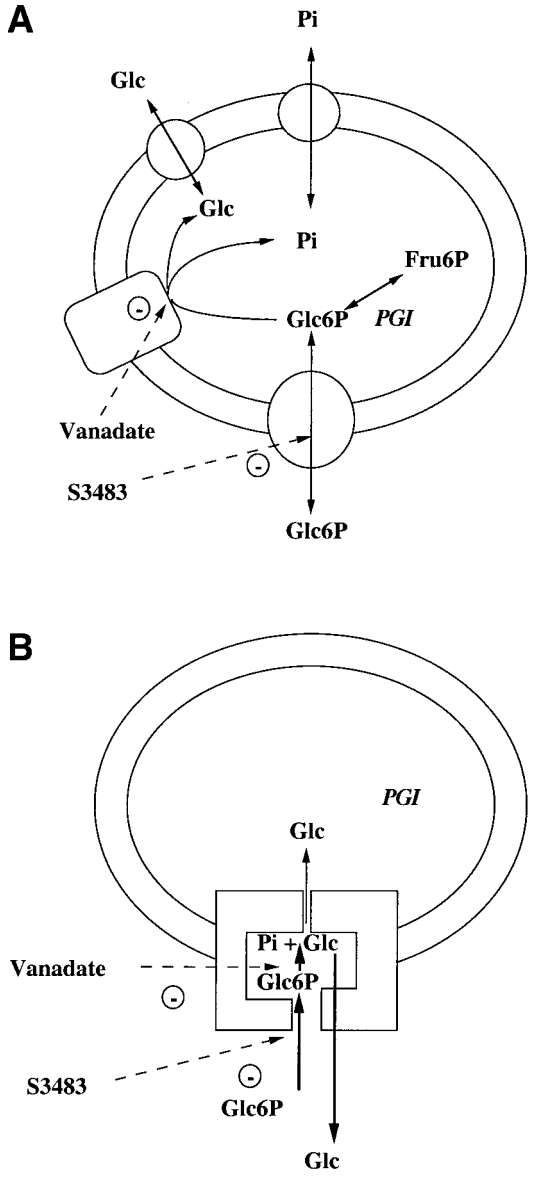

FIG. 1. Models for glucose-6-phosphatase and principle of the method used to demonstrate glucose-6-phosphate transport. *A*: According to the substrate-transport model, glucose-6-phosphate (Glc6P) is transported into the lumen of the endoplasmic reticulum, where it is hydrolyzed by glucose-6-phosphatase to glucose (Glc) and Pi. These are then transported out of the vesicles by appropriate transporters. *B*: According to the conformational model of Annabi and van de Werve (15), the hydrolysis of glucose-6-phosphate is an intramembranous event with release of the products occurring mainly on the cytosolic side of the membrane. Vanadate inhibits the phosphohydrolase in both models. The chlorogenic acid derivative S3483 inhibits glucose-6-phosphatase by blocking glucose-6-phosphate transport in model *A* and in an undefined way in model *B*. Only in the first model does one expect that microsomes loaded with phosphoglucose isomerase (PGI) catalyze the detritiation of [2-^{3}H]glucose 6-phosphate in a S3483-inhibitable manner.

doplasmic reticulum is not needed for its hydrolysis by glucose-6-phosphatase (15,17). In addition, uptake of radiolabeled glucose-6-phosphate by microsomes is reduced when they are derived from the liver of patients with GSD Ia (18), from mouse glucose-6-phosphatase knock-outs, or when glucose-6-phosphatase from normal liver is inhibited by vanadate (19), suggesting that transport of glucose 6-phosphate is dependent on glucose-6-phosphatase activity. These observations led to the development of a model (conformational model, Fig. 1*B*) according to which glucose-6-phosphate hydrolysis occurs in the membrane per se rather than in the lumen of the endoplasmic reticulum (15).

A major difficulty in measuring transport of glucose-6-phosphate into microsomes stems from the fact that they are very small structures with a high surface-to-volume ratio. Therefore, transported molecules may cross the membrane backwards in a matter of seconds during the washing step needed to remove nontransported molecules (5,20). By the same token, a correct assessment of the proportion of glucose and Pi first released in the lumen of the endoplasmic reticulum upon hydrolysis of glucose-6-phosphate may be prevented by the rapid efflux of these products from the microsomes (5).

In the present study, we have addressed two questions. The first one concerns the presence in microsomal membranes of a glucose-6-phosphate transporter that is sensitive to S3483 and acts independently of glucose-6-phosphatase. To this end, we took advantage of the fact that phosphoglucose isomerase catalyzes the detritiation of [2-^{3}H]glucose-6-phosphate (21) and introduced this enzyme into microsomes. In the context of the substrate-transport model, but not in the case of the intramembrane hydrolysis model, such microsomes are expected to catalyze the detritiation of [2-^{3}H]glucose-6-phosphate in a S3483-sensitive manner. The second question concerns the site where the products of the glucose-6-phosphatase reaction are first released. To prevent rapid leakage of Pi from the lumen of the microsomes, we precipitated this product in situ as its Pb^{2+} salt, a procedure that has been previously used to qualitatively demonstrate the formation of intravesicular Pi (22) but has not been used to quantify this formation. We also took advantage of the ability of glucose-6-phosphatase to convert mannitol- and glucose-6-phosphate into mannitol-1-phosphate (23), a nonphysiological compound for which there is presumably no specific transporter.

RESEARCH DESIGN AND METHODS

Materials. Yeast phosphoglucose isomerase, hexokinase, and glucose-6-phosphate dehydrogenase were from Roche Molecular Biochemicals. Dowex 1×8 chloride and borate resins were purchased from Acros. Poly(ethyleneglycol) 6,000 was from UCB (Belgium) and leupeptin and antipain from Takara (Japan). All other reagents (analytical grade) were from Acros or Sigma. [2-^{3}H]glucose-6-phosphate was prepared by phosphorylation of [2-^{3}H]glucose by hexokinase and purified by chromatography on Dowex 1×8 chloride and elution with an NaCl step gradient. [^{32}P]glucose-6-phosphate was synthesized as described by Vandercammen et al. (24). S3483 was kindly made available to us by Dr. H.J. Burger (Aventis Pharma Deutschland).

Entrapment of phosphoglucose isomerase in microsomes. Washed liver microsomes (0.8–1.0 mg protein) prepared from overnight-fasted male Wistar rats were mixed with (final concentrations) 10 mmol/l Hepes (pH 7.1), 100 mmol/l KCl, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 mmol/l octylglucoside, and 6 U yeast phosphoglucose isomerase (Roche) in a final volume of 50 μl. The mixture was frozen in dry ice for 10 min, thawed at 30°C for 10 min, and thoroughly vortexed. After two additional freezing and thawing cycles, the preparation was mixed with 1.5 ml of a buffer (buffer A) containing 10 mmol/l Hepes (pH 7.1), 100 mmol/l KCl, and 1 mg/ml defatted bovine serum albumin. After 10 min at room temperature, $CaCl_2$ and poly(ethyleneglycol) were added to final concentrations of 0.5 mmol/l and 2%, respectively. The microsomes were spun down by a 5-min centrifugation at 10,000*g* at room temperature. They were then washed three times in 1.5 ml buffer A containing 0.5 mmol/l $CaCl_2$ and 2% poly(ethyleneglycol). The last pellet was resuspended in 35 μl of a 10 mmol/l Hepes buffer (pH 7.1), containing 100 mmol/l KCl and 1 mmol/l dithiothreitol. Phosphoglucose isomerase assays showed that the four successive supernatants contained ~90, ~1, 0.2, and <0.1%, respectively, of the original phosphoglucose isomerase activity, and the final pellet contained ~2.0–3.5%. Phosphoglucose isomerase was assayed spectrophotometrically

at 30°C in a reaction mixture containing 100 mmol/l Tris (pH 7.5), 7.5 mmol/l $MgCl_2$, 0.25 mmol/l NADP, 5 μg/ml yeast glucose-6-phosphate dehydrogenase, and 1 mmol/l fructose-6-phosphate. One unit was the amount catalyzing the formation of 1 μmol glucose-6-phosphate per min under these conditions.

Measurements of detritiation and glucose release. The measurements of detritiation were carried out at 30°C in a 200-μl mixture containing 20 mmol/l Hepes (pH 7.1), 100 mmol/l KCl, 1 mmol/l dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.2 mmol/l glucose-6-phosphate, 100,000 cpm [2-^{3}H]glucose-6-phosphate, and 2 μl of the microsomal preparation containing ~10 mU phosphoglucose isomerase as well as the indicated concentrations of inhibitors. The reaction was stopped after 20 min by the addition of 0.1 ml 10% perchloric acid. After centrifugation, the supernatant was neutralized with 3 mol/l K_2CO_3 and tritiated water isolated on Dowex 1×8 columns, borate forms (25), and counted. In some experiments, an aliquot of the supernatant was also applied on Dowex 1×8 column, chloride form (1 ml of resin, in a Pasteur capillary pipette), which was washed with 2 ml water to elute [^{3}H]water and [^{3}H]glucose. [^{3}H]glucose was calculated by subtracting the proportion of radioactivity eluted from the borate column from that eluted from the chloride column.

Determination of intravesicular Pi. Microsomes (~150 μg protein) were incubated at 30°C in 50 mmol/l Mes (pH 6.5), 100 mmol/l KCl, 2 mmol/l $MgCl_2$, 4% poly(ethyleneglycol), 1 mmol/l glucose-6-phosphate, and 100,000 cpm [^{32}P]glucose-6-phosphate with or without 0.5 mmol/l $Pb(acetate)_2$ in a final volume of 0.2 ml. At the indicated times, EDTA was added to a final concentration of 10 mmol/l, and the samples were submitted 30 s later to a short centrifugation (15 s at 10,000*g*). Perchloric extracts were prepared from the pellets and the supernatants. After neutralization, the extracts were diluted threefold with water and applied onto anion-exchange columns (Dowex 1×8 Cl^- resin), which were washed with 2 ml water. [^{32}P]Pi was eluted with 1.5 ml 0.5 mol/l NaCl and further isolated as a phosphomolybdate complex (26) before being counted. The chromatographic step was needed to remove poly(ethyleneglycol), which interfered in the extraction of the phosphomolybdate complex.

Formation of mannitol-1-phosphate. Microsomes (700 μg/ml) were incubated at 30°C in the presence of 50 mmol/l Hepes (pH 7.1), 100 mmol/l KCl, 10 mmol/l glucose-6-phosphate, 200 mmol/l mannitol, and 4% poly(ethyleneglycol) in a final volume of 0.4 ml. At the indicated times, vanadate was added to a final concentration of 1 mmol/l, and the samples were immediately centrifuged. The resulting supernatant was mixed with 0.2 ml 10% perchloric acid, and the pellet was resuspended in 0.2 ml 10% perchloric acid. This arrest procedure took <30 s. Mannitol 1-phosphate was measured enzymatically (23).

RESULTS

Entrapment of phosphoglucose isomerase into microsomes. The method we wanted to use for the measurement of glucose-6-phosphate transport into microsomes requires the presence of phosphoglucose isomerase in the lumen of these vesicles. However, like other glycolytic enzymes, phosphoglucose isomerase is essentially present in the cytosol. Therefore, we developed a procedure to entrap purified yeast phosphoglucose isomerase into microsomes. This procedure consisted of mixing the microsomes with phosphoglucose isomerase in the presence of octylglucoside and submitting the preparation to freezing and thawing cycles. Four washings were then performed in a medium that contained $CaCl_2$ and poly(ethyleneglycol) to facilitate sedimentation of the microsomes.

Figure 2 illustrates this procedure applied to liver microsomes. Phosphoglucose isomerase was assayed both in the absence and presence of Triton X-100, which reveals the latent (membrane-surrounded) enzyme. The use of 10 mmol/l octylglucoside and three freezing and thawing cycles was found to be optimal for liver microsomes, resulting in the incorporation of ~2.0–3.5% of the initial phosphoglucose isomerase, with a latency >75%. Lower degrees of incorporation were observed in the absence of octylglucoside or when no or only one freezing and thawing cycle was performed. Slightly higher degrees of incorporation were observed when 40 mmol/l octylglucoside was used instead of 10 mmol/l, but in this case, <20% of the total activity was latent.

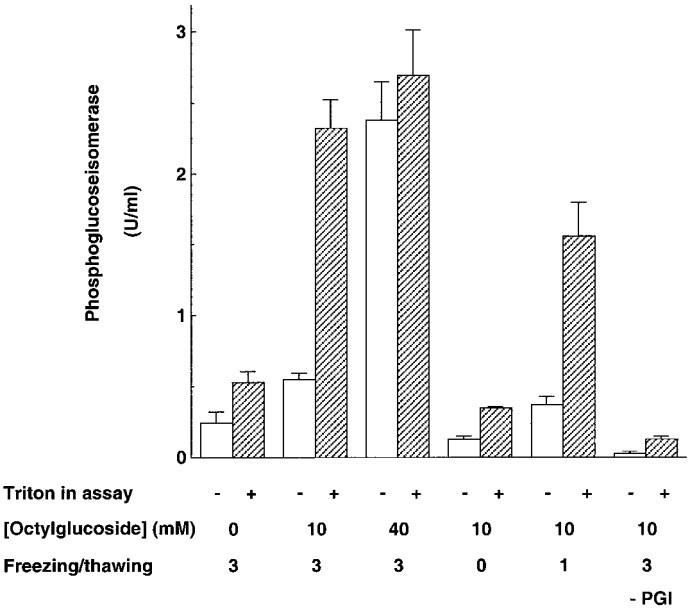

FIG. 2. Conditions required for loading microsomes with phosphoglucose isomerase. Rat liver microsomes (25 μl of 50 mg protein/ml) were mixed with 5 U yeast phosphoglucose isomerase and 0, 10, or 40 mmol/l octylglucoside in a final volume of 50 μl. The mixture was submitted to zero, one, or three freezing/thawing cycles, as indicated. The microsomes were washed and centrifuged four times; the final pellet was resuspended in 40 μl, and the phosphoglucose isomerase activity was measured in the absence or presence of 0.1% Triton X-100. A control performed without yeast phosphoglucose isomerase is also shown (-PGI). The results shown are the means of three experiments ± SEM.

We also evaluated the effect of such treatments on the inhibition exerted by 50 μmol/l S3483 on glucose-6-phosphatase. This inhibition amounted to 90% in untreated microsomes but to only ~40% in the microsomes that had been freeze-thawed three times in the presence of 10 mmol/l octylglucoside, either with or without phosphoglucose isomerase. This decreased inhibition may be partly explained by membrane eversion (see DISCUSSION).

Effect of S3483 and PGI inhibitors on detritiation. We first checked that detritiation of [2-^{3}H]glucose-6-phosphate by purified phosphoglucose isomerase was not inhibited by 50 μmol/l S3483. Because part of the phosphoglucose isomerase in the microsomal preparation was not latent, it was of interest to use inhibitors of phosphoglucose isomerase that would be excluded from the vesicles derived from endoplasmic reticulum and therefore preferentially inhibit nonlatent phosphoglucose isomerase. Two such compounds were used, erythrose-4-phosphate and fructose-1,6-bisphosphate, both of which act as competitive inhibitors (27; E.V.S., unpublished results). Under our experimental conditions, they caused 50% inhibition of detritiation by purified yeast phosphoglucose isomerase at 8 μmol/l and 0.4 mmol/l, respectively. Their effect was not modified by the presence of 50 μmol/l S3483 (data not shown).

Figure 3 illustrates the effects of S3483, erythrose-4-phosphate and fructose-1,6-bisphosphate on the detritiation catalyzed by microsomes loaded with phosphoglucose isomerase. In this first type of experiment, glucose-6-phosphatase was inhibited by 1 mmol/l vanadate (28) to facilitate interpretation of the results. In the absence of erythrose-4-phosphate or fructose-1,6-bisphosphate, S3483

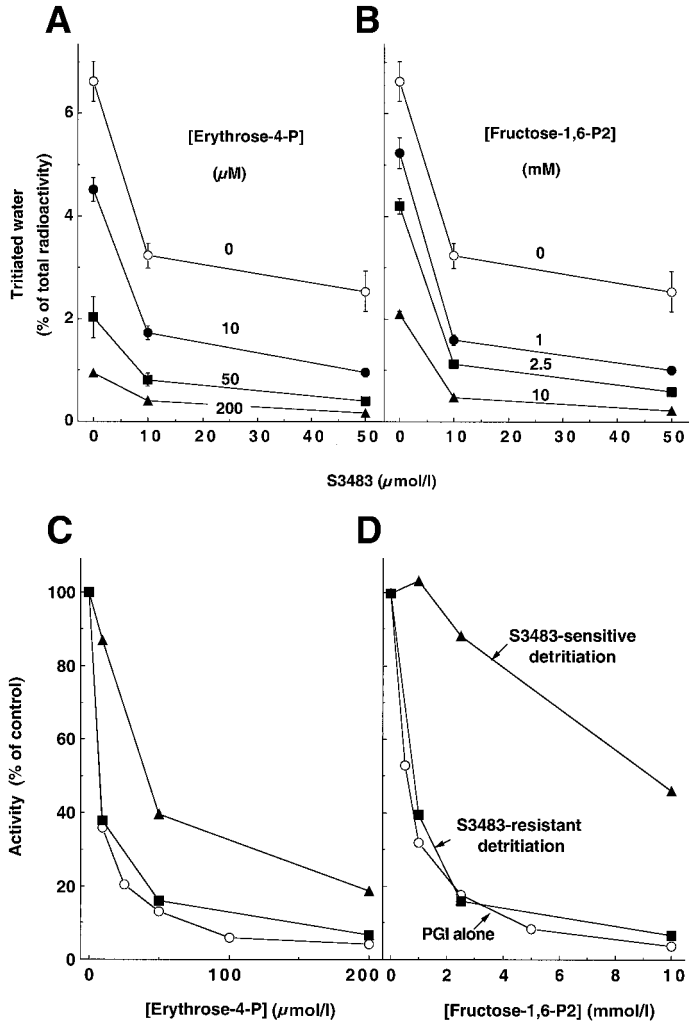

FIG. 3. Effect of S3483, erythrose-4-phosphate (*A*), and fructose-1,6-bisphosphate (*B*) on the detritiation catalyzed by phosphoglucose isomerase entrapped into liver microsomes. Rat liver microsomes loaded with phosphoglucose isomerase were incubated for 15 min with [2-^{3}H]glucose-6-phosphate in the presence of the indicated concentrations of S3483, erythrose-4-phosphate, and fructose-1,6-bisphosphate. In *C* and *D*, the S3483-sensitive (difference between the values obtained without and with 50 μmol/l S3483) and S3483-resistant (values observed with 50 μmol/l S3483) detritiation have been replotted and compared with the detritiation catalyzed by purified phosphoglucose isomerase (PGI alone). The results shown are the means of three experiments ± SEM.

inhibited detritiation by up to ~60% at a concentration of 50 μmol/l, whereas in the presence of these phosphate esters, the inhibition increased up to ~90%. These results indicated that phosphoglucose isomerase entrapped into liver microsomes had become sensitive to the putative inhibitor of glucose-6-phosphate transport.

Replot of the data shown in Fig. 3*A* and *B* showed that the S3483-sensitive component of detritiation was approximately five times less sensitive to erythrose-4-phosphate and 10 times less sensitive to fructose-1,6-bisphosphate than the detritiation catalyzed by microsomes in the presence of a saturating concentration of S3483 or that catalyzed by free phosphoglucose isomerase (Fig. 3*C* and *D*). This result indicated that erythrose-4-phosphate and fructose-1,6-bisphosphate have no free access to the lumen of microsomes.

Effect of vanadate on detritiation. We then explored the effect of vanadate with or without fructose-1,6-bisphosphate and S3483 both on the hydrolysis and the detritiation of [2-^{3}H]glucose-6-phosphate. If glucose-6-phosphatase and phosphoglucose isomerase competed for one single intramicrosomal glucose-6-phosphate pool, inhibition of the phosphatase might produce an increase in the detritiation rate. Vanadate, at a concentration (1 mmol/l) that inhibited glucose-6-phosphatase activity almost completely, stimulated the release of tritiated water in the absence but not in the presence of S3483 (Fig. 4), indicating that it acted only on the luminal component of detritiation. The stimulation of the S3483-sensitive component of detritiation amounted to 1.5-fold in the absence of fructose-1,6-bisphosphate and to approximately twofold in its presence. We also noted that fructose-1,6-bisphosphate had a modest stimulatory effect on glucose-6-phosphatase at all concentrations of S3483.

Evidence for the intravesicular formation of Pi. The goal of this second set of experiments was to quantify the proportion of the Pi formed by glucose-6-phosphatase that is initially present in the lumen of liver microsomes. To this end, we carried out incubations in the presence of Pb^{2+} to precipitate Pi in situ and hence slow down its release from microsomes. The concentration of Pb(acetate)$_2$ we used (0.5 mmol/l) had minimal inhibitory effect on glucose-6-phosphatase activity. When mixed with 0.1 mmol/l [^{32}P]Pi, it caused the instantaneous (in <30 s) and quantitative formation of a complex, which rapidly sedimented upon centrifugation (15 s at 10,000*g*). Addition of 10 mmol/l EDTA to such a complex caused its instantaneous (<30 s) solubilization, so that no radioactivity sedimented any longer upon centrifugation.

In the experiment shown in Fig. 5, microsomes were incubated with 1 mmol/l [^{32}P]glucose-6-phosphate and 4% poly(ethyleneglycol) (to facilitate sedimentation of the vesicles) either with or without 0.5 mmol/l Pb^{2+}. At the indicated times, 10 mmol/l EDTA was added to the reaction mixture to dissolve all extramicrosomal precipitates,

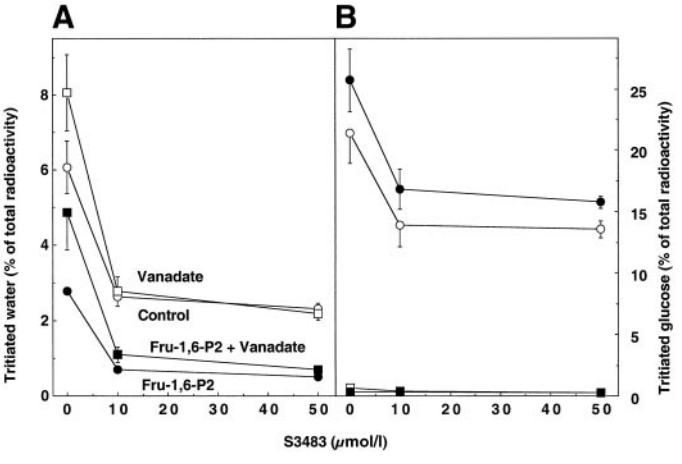

FIG. 4. Effect of vanadate, fructose-1,6-bisphosphate, and S3483 on the release of radioactive water (*A*) and glucose (*B*) from [2-^{3}H]glucose-6-phosphate by liver microsomes loaded with phosphoglucose isomerase. Rat liver microsomes were incubated for 15 min with the indicated concentrations of S3483 with (□, ■) or without (○, ●) 1 mmol/l vanadate, and 0 (open symbols) or 1 (closed symbols) mmol/l fructose 1,6-bisphosphate. Neutralized perchloric extracts were chromatographed on Dowex 1×8 borate to isolate [^{3}H]H_2O and on Dowex 1×8 chloride to isolate [^{3}H]H_2O and [^{3}H]glucose. The latter was calculated by subtraction. The results shown are the means of three experiments ± SEM.

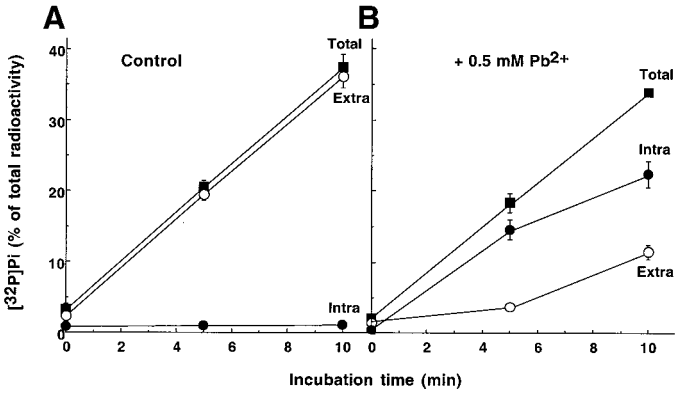

FIG. 5. Intravesicular and extravesicular [^{32}P]Pi formed from [^{32}P]glucose-6-phosphate in the presence and absence of Pb^{2+}. Rat liver microsomes were incubated at 30°C with 1 mmol/l [^{32}P]glucose-6-phosphate, 4% poly(ethyleneglycol), without 0.5 mmol/l lead acetate (*A*) or with 0.5 mmol/l lead acetate (*B*). At the indicated times, EDTA was added to a final concentration of 10 mmol/l; the samples were centrifuged 30 s later, and Pi was isolated from perchloric extracts prepared from the supernatants (extra) and the pellets (intra). The results shown are the means of three experiments ± SEM.

and the samples were centrifuged 30 s later. Supernatants (containing the medium) and pellets (containing the microsomes) were rapidly separated and treated with perchloric acid. After neutralization, inorganic phosphate was isolated and counted. Total formation of Pi by glucose-6-phosphatase was linear with time and was only minimally (~10%) inhibited by Pb^{2+} (Fig. 5). In the absence of Pb^{2+}, essentially all Pi was found in the medium, whereas in its presence (Fig. 5*B*), 80% of this product was recovered in the microsomal pellet after 5 min and 65% after 10 min.

To verify that the lead phosphate precipitate was surrounded by a membrane, we tested the effect of Triton X-100 with or without EDTA on its solubilization. As shown in Fig. 6, a combination of Triton X-100 (0.1%) and EDTA (10 mmol/l) caused the instantaneous dissolution of the precipitate, whereas these agents had minimal effects when used separately.

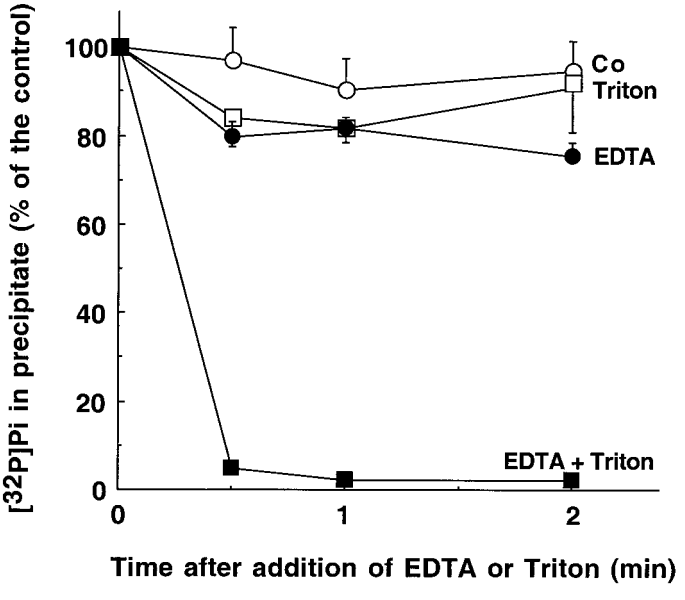

FIG. 6. Effect of EDTA and Triton X-100 on the lead phosphate complex formed upon hydrolysis of glucose-6-phosphate by glucose-6-phosphatase. Rat liver microsomes were preincubated for 10 min at 30°C with 1 mmol/l [^{32}P]glucose-6-phosphate, 4% poly(ethyleneglycol), and 0.5 mmol/l lead acetate. EDTA (10 mmol/l) and Triton X-100 (0.1%) were then added either alone or in combination. Samples were centrifuged at the indicated times after these additions, and Pi was isolated from perchloric acid extracts prepared from the pellets. The results shown are the means of three experiments ± SEM.

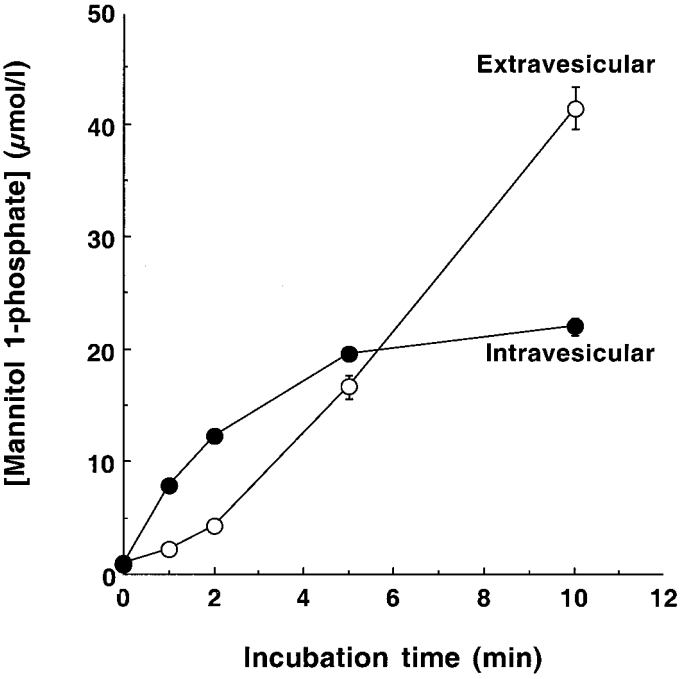

FIG. 7. Distribution of the mannitol-1-phosphate formed upon incubation of liver microsomes with glucose-6-phosphate and mannitol. Microsomes were incubated in the presence of glucose-6-phosphate and mannitol. At the indicated times, the reaction mixture was centrifuged, and perchloric acid extracts were prepared from the supernatants and pellets for determination of extravesicular and intravesicular mannitol-1-phosphate. The results shown are the means of three experiments ± SEM.

Evidence for the intravesicular formation of mannitol-1-phosphate. The problem of the primary site of release of the products of the glucose-6-phosphatase reaction was also investigated by measuring the formation of mannitol-1-phosphate from mannitol- and glucose-6-phosphate. Figure 7 shows that mannitol-1-phosphate first appeared inside the microsomes to reach a plateau after ~5 min, whereas the appearance of this polyol-phosphate in the medium was delayed. Quite significantly, ~80% of the mannitol-1-phosphate that had been formed at the shortest investigated time (1 min) was intravesicular. Other experiments showed that 50 μmol/l S3483 inhibited the total formation of mannitol-1-phosphate by ~85% when glucose-6-phosphate was the phosphate donor (results not shown).

DISCUSSION

Loading of microsomes with phosphoglucose isomerase. Our procedure to entrap phosphoglucose isomerase into microsomes allowed the incorporation of ~2.0–3.5% of the initial isomerase activity. This represents ~50% of the maximal theoretical incorporation that could be expected. The latter roughly amounts to the ratio of intravesicular water to total water in the incubation mixture, i.e., 5.1–6.4% if one assumes a microsomal protein concentration of 16–20 mg/ml in the incubation medium and an

intracellular water space of 3.2 μl/mg protein (29). The high degree of enzyme incorporation indicates that phosphoglucose isomerase has been introduced in the lumen of the endoplasmic reticulum.

Part of the isomerase that was associated with the microsomes (typically 10–20% in liver microsomes prepared with our standard procedure) was not latent. This problem is most likely not caused by external phosphoglucose isomerase that remains adsorbed to the microsomes because the microsomal preparations were extensively washed after enzyme incorporation (see RESEARCH DESIGN AND METHODS). Furthermore, the proportion of isomerase that was not latent was quite high (~85%) when the microsomes had been treated with a higher concentration (40 mmol/l) of octylglucoside. Therefore, we think that the nonlatent activity is contributed by microsomes that have incorporated phosphoglucose isomerase but are not tightly resealed because of residual detergent.

A second problem was that the loading treatment decreased (from 90 to ~40%) the inhibition exerted by S3483 on glucose-6-phosphatase as well as the latency of mannose-6-phosphatase (data not shown). Both changes may be partly caused by the presence of leaky microsomes but also partly by eversion of the microsomal membrane. In the context of the substrate-transport model, this should lead to externalization of the catalytic site of glucose-6-phosphatase and loss of sensitivity to inhibitors of glucose-6-phosphate transport. S3483 is nonetheless expected to inhibit isomerization of glucose-6-phosphate catalyzed by such inverted vesicles because phosphoglucose isomerase is inside. The fact that S3483 inhibited more detritiation of [2-^{3}H]glucose-6-phosphate than its hydrolysis (Fig. 4) indicates the presence of such inside-out vesicles.

Despite these problems, the phosphoglucose isomerase-loaded vesicles were a valid model to study glucose-6-phosphate entry, as further discussed below.

Independence of glucose-6-phosphate transport and hydrolysis. The observation that the inclusion of phosphoglucose isomerase into microsomes made it sensitive to inhibition by S3483 is best explained by assuming that this compound inhibits the entry of glucose-6-phosphate into microsomes. The fact that erythrose-4-phosphate and fructose-1,6-bisphosphate were 5- to 10-fold less potent to inhibit the S3483-sensitive (intramicrosomal) detritiation than that catalyzed by free phosphoglucose isomerase indicates that these compounds do not readily penetrate the intramicrosomal space. Therefore, these two pieces of evidence support the existence of a specific glucose-6-phosphate transporter that transfers its substrate from the surrounding medium to the lumen of the microsomes (and reciprocally because this transport is not energy-driven).

Previous experiments have shown that the transport of [^{14}C]glucose-6-phosphate is apparently inhibited by vanadate, or when glucose-6-phosphatase is inactive due to mutations in its gene (18,19). These experiments were interpreted as showing that glucose-6-phosphate transport and hydrolysis are linked, i.e., that glucose-6-phosphate transport occurs concomitantly with its hydrolysis in the membrane of the endoplasmic reticulum. Quite the contrary, we show that vanadate caused a 1.5- to 2.0-fold stimulation of the detritiation of [2-^{3}H]glucose-6-phosphate in microsomes loaded with phosphoglucose isomerase. This effect indicates that glucose-6-phosphatase and phosphoglucose isomerase compete for a single intramicrosomal substrate pool (as in the substrate-transport model) and that the glucose-6-phosphate transporter is rate-limiting under our experimental conditions.

As pointed out by Banhegyi et al. (5), the results of St-Denis et al. (18) and Lei et al. (19) can be reconciled with the substrate-transport model by considering that, when microsomes are incubated with [^{14}C]glucose-6-phosphate, most (often close to 90%) of the radioactivity that accumulates in the vesicles (or most likely in a small fraction of them that have no rapid mechanism for glucose extrusion) corresponds to [^{14}C]glucose, formed by hydrolysis of glucose-6-phosphate. Inhibition or inactivation of glucose-6-phosphatase decreases this formation, thereby decreasing the apparent uptake of [^{14}C]glucose-6-phosphate.

Luminal orientation of the catalytic site of glucose-6-phosphatase. The first piece of direct evidence for the orientation of the catalytic site of glucose-6-phosphatase toward the lumen of the endoplasmic reticulum came from the observation of luminal accumulation of lead phosphate by electron microscopy in sections of microsomes incubated with glucose-6-phosphate (22). We have now exploited the same principle to reach a quantitative assessment of the proportion of Pi that is formed inside microsomes. We found indeed that at the shortest time investigated, at least 80% of the hydrolyzed Pi can be precipitated with Pb^{2+} in membrane-bound vesicles. This intramicrosomal localization was indicated by the fact that the precipitate could be efficiently released only in the combined presence of a metal ion chelator and a detergent, whereas a simple lead phosphate precipitate only needed a chelator to be dissolved. Calculations indicate that the microsomal phosphate precipitate represented, after 10 min, ~160 nmol Pi/mg microsomal protein. Such an amount is compatible with a luminal location (corresponding then to a concentration of ~50 mmol/l, assuming an intravesicular water space of 3.2 μl/mg protein), but is difficult to reconcile with an intramembrane location (in a pocket in close proximity to the catalytic site, as in the conformational model). The observation that a minor part of the formed Pi was not precipitated with Pb^{2+} is most likely caused by the rate-limiting aspect of Pb^{2+} entry into microsomes as well as by the fact that a small proportion of the vesicles were not latent.

Another argument in favor of the luminal orientation of the catalytic site came from the observation that the majority (at least 80%) of the mannitol-1-phosphate that was produced from glucose-6-phosphate and mannitol was initially intramicrosomal. Under our experimental conditions, the steady-state intramicrosomal content reached 25 nmol/mg protein, corresponding to a luminal concentration of ~8 mmol/l. Release of this phosphate ester from the vesicles at longer times indicates the presence of a transporter that slowly acts on mannitol-1-phosphate.

Although we do not provide evidence in this work that the primary site of release of glucose by glucose-6-phosphatase is the lumen of the endoplasmic reticulum, there are several reasons to think that such is the case. The first reason is that it is difficult to conceive how the catalytic site of glucose-6-phosphatase, an integral membrane pro-

tein (30), would be able to deliver some of its products (Pi, mannitol-1-phosphate) in the lumen of the endoplasmic reticulum and another product, glucose, in an intramembrane pocket. A second reason is the fact that S3483 inhibits a transporter that transfers glucose-6-phosphate to the lumen of the endoplasmic reticulum (see above), indicating that this substrate of glucose-6-phosphatase accesses the catalytic site via the luminal side of the membrane. The reason why most of the glucose that is formed from glucose-6-phosphate appears to be immediately in the medium is the same reason why it is true for Pi: transport from most vesicles is so rapid that, unless there is an efficient way of blocking it (such as Pb^{2+} in the case of Pi), released products are exported in a matter of seconds, preventing any correct assessment of their site of production. There is indeed ample evidence that a majority of the microsomal vesicles are equipped with transporters or pore-like structures that allow them to equilibrate glucose across their membrane in a matter of seconds (5,20).

This rapid equilibration of glucose across the membrane of the endoplasmic reticulum strongly argues against a role of vesicle-mediated secretion of glucose by the normal liver. Such a mechanism was postulated to account for the fact that mice that have no GLUT2 in their liver, having normal glucose output (31). An alternative explanation could be that other glucose transporters could take over the role of GLUT2, though less efficiently than the latter.

In conclusion, we have provided three new and independent arguments supporting the glucose-6-phosphatase model initially proposed by Arion et al. (2). These arguments concur with other kinetic evidence (2), with measurements of metabolite transport (5), and with topological studies of glucose-6-phosphatase (30). They agree also with the observation that GSD Ib, in which there is a defect in microsomal glucose-6-phosphate transport, is caused by mutations in a protein member of the organophosphate transporter family (13,32,33).

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