The glucose-6-phosphate transport is not mediated by a glucose-6-phosphate/phosphate exchange in liver microsomes

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Abstract
A phosphate-linked antiporter activity of the glucose-6-phosphate transporter (G6PT) has been recently described in liposomes including the reconstituted transporter protein. We directly investigated the mechanism of glucose-6-phosphate (G6P) transport in rat liver microsomal vesicles. Preloading with inorganic phosphate (Pi) did not stimulate G6P or Pi microsomal inward transport. Pi efflux from pre-loaded microsomes could not be enhanced by G6P or Pi addition. Rapid G6P or Pi influx was registered by light-scattering in microsomes not containing G6P or Pi. The G6PT inhibitor, S3483, blocked G6P transport irrespectively of experimental conditions. We conclude that hepatic G6PT functions as an uniporter.

1. Introduction
The glucose-6-phosphate transporter (G6PT, also known as SLC37A4) is an integral endoplasmic reticulum (ER) protein, which mediates the entry of the cytosolic metabolite glucose-6-phosphate (G6P) into the ER lumen. In the ER lumen, G6P is the substrate of at least two enzymes: the glucose-6-phosphatase (EC 3.1.3.9., G6Pase), and the hexose-6-phosphate dehydrogenase (EC 1.1.1.47., H6PD). The G6PC isoform of the former enzyme is highly expressed in the glucogenic organs liver and kidney and regulates blood glucose homeostasis; other isoforms, namely G6PC2 and G6PC3, are expressed in various non glucogenic tissues. The inherited deficiency of G6PC and G6PT causes glycogen storage disease type 1 a and type 1 non-a, respectively, since G6P hydrolysis is prevented, particularly in glucogenic tissues.

Original evidence in liver ER-derived vesicles, i.e. microsomes, suggested that G6PT works as a facilitative bi-directional transporter. More recently, it has been shown that G6PT, once overexpressed in COS-1 cells and subsequently reconstituted in liposomes, behaves as a phosphate-linked antiporter. In view of this discrepancy, in the present paper we have directly investigated this point. In particular, we aimed at evidence the possible G6P-phosphate antiport activity of G6PT in native rat liver microsomes. We assumed this model to reflect the physiological conditions of the liver ER. Indeed, microsomal vesicles from the liver have been considered for a long time an equivalent of ER in vitro and have been used in a large number of studies related to ER transport. The present results compellingly indicate that in liver microsomes G6PT does not operate as an antiporter. We conclude that liver G6PT works as a facilitative bidirectional G6P uniporter.

2. Materials and methods

2.1. Materials
G6P (dipotassium salt), mannose-6-phosphate (disodium salt), alamethicin, NADPH, and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) were from Sigma. Na$_3$VO$_4$ was from Fisher Science Co. S3483 was a gift of Aventis Pharma, Frankfurt, Germany.
7.2. The suspensions were rapidly frozen and maintained under 
filter membranes (pore size 0.22 μm) [7]. To distinguish the intravesicular 
radioactivity was measured as intravesicular [7]. The alamethicin-
treated microsomes retained amounts of radioactivity 
radioactive aliquots (0.1 ml) were used for rapid filtration measurements as de-
tailed below.

2.3. Loading of microsomes with inorganic phosphate anions 
In some experiments, microsomes (20 mg protein/ml) were 
incubated in the KCl/Mops buffer pH 7.2 in the presence of 5 mM 
K-phosphate buffer (pH 7.2) for 1 h at 22 °C. In other experiments, 
to increase the intravesicular phosphate concentration, microsomal vesicles (20 mg protein/ml) in the KCl/Mops buffer pH 7.2 were 
opened/resealed by freezing/thawing three times in the presence 
of 5 mM K-phosphate buffer (pH 7.2), essentially as reported [8], except that suspensions were frozen in liquid nitrogen for 
5 min and subsequently thawed 5 min at 37 °C, for each cycle, and no detergent was used.

The amount of intravesicular inorganic phosphate (Pi) was eval-
uated by adding trace amounts of 32P to the microsomal suspensions before incubating or the freezing/thawing procedure; aliquots (20 μl) were used for rapid filtration measurements as de-
tailed below.

2.4. Uptake of G6P 
After Pi pre-loading, microsomal suspensions were immediately 
20-fold diluted in the KCl/Mops buffer pH 7.2 containing the de-
sired amounts of G6P plus 32Pi (24 μCi/ml). At the indicated 
time intervals, aliquots (0.1 ml) were rapidly withdrawn and filtered through cellulose acetate/nitrate filters (pore size 0.22 μm) and washed with 4 ml of Hepes (20 mM) buffer pH 7.2 containing 250 mM sucrose and 1 mM DIDS. The radioactivity associated with microsomes retained by filters was measured by li-
quid scintillation counting [7]. To distinguish the intravesicular and the bound radioactivity, alamethicin (0.1 mg/ml) was added to parallel incubates and the alamethicin–releasable portion of radioactivity was regarded as intravesicular [7]. The alamethicin-
treated microsomes retained amounts of radioactivity ≤15% of that 
associated to untreated microsomes.

2.5. Release/uptake of inorganic phosphate 
For evaluating the microsomal release of Pi, microsomes were treated by freezing/thawing in the presence of 5 mM K-phosphate buffer pH 7.2 plus 32Pi (75 μCi/ml) and then 20-fold diluted in the KCl/Mops buffer pH 7.2 containing 5 mM K-phosphate plus 32Pi (75 μCi/ml). At the indicated time intervals, aliquots (0.1 ml) were rapidly withdrawn and Pi vesicular content was measured as de-
scribed above.

2.6. Light-scattering measurement of the microsomal permeability to G6P and Pi 
The permeability of the microsomal membranes towards G6P and Pi was measured by the continuous detection of the osmotically 
induced changes in size and shape of microsomal vesicles by light-
scattering [6 and Refs. therein]. Microsomal vesicles (20 mg protein/ml) were frozen and thawed in a hypotonic medium (5 mM 1,4-pip-
erazinediethanesulfonic acid potassium salt, pH 7.0) to release intravesicular components, recovered by rapid centrifugation [8], 
resuspended in the hypotonic medium to have 100 μg protein per
ml, and equilibrated for 3 h at 22 °C. Light-scattering of microsomal suspensions was monitored at 400 nm at right angles to the incoming 
light beam using a Cary Eclipse fluorescence spectrophotometer (Varian), equipped with a temperature-controlled cuvette holder (22 °C) and magnetic stirrer. Vesicle shrinking (increased light-scat-
tering) and swelling (decreased light-scattering) were detected after the addition of a small volume (<5% of the total incubation vol-
ume) of concentrated and neutralised solutions of the compounds to be tested. Light-scattering intensity was acquired each 0.2 s.
The freezing/thawing procedure did not modify the pattern in light scattering signal as verified in preliminary experiments.

2.7. Other assays 
Intactness of microsomal vesicles checked by measuring the la-
tency of mannose-6-phosphatase activity [7] was greater than 90% 
independently of any treatment employed. Glucose-6-phosphatase 
activity was measured as reported [7]. Pi content of microsomal vesicles was measured according to [12].

2.8. Statistical analysis 
Results are expressed as means ± S.D. Results were compared using the Student’s two-tailed t-test: P < 0.05 was considered sta-
tistically significant.

3. Results 
3.1. Uptake of radiolabeled G6P by microsomes pre-loaded with Pi 
To evidence a putative G6P–Pi antiport activity, a concentrated suspension of liver microsomes (20 mg of protein per ml) was pre-
loaded with Pi by incubation in the presence of 5 mM K-phosphate buffer pH 7.2, for 1 h. The incubation resulted in the loading of microsomal vesicles of 7.1 nmol Pi per mg of protein. Assuming a vesicular water space of 3.1 μl/mg protein, and an evenly distribution of the anion in microsomal vesicles, an intravesicular Pi concentration of 2.3 mM could be calculated. The uptake of G6P was 
started by a 20-fold-dilution of the microsomal suspension in the Pi-free buffer containing 1 mM G6P (and the [14C(U)] G6P tracer). The dilution creates a gradient between intra- and extra-vesicular Pi concentration (2.3 mM versus 0.25 mM), which is expected to favour G6P uptake in the case that a G6P–Pi antiport is operative. By contrast, the G6P uptake was not increased in Pi loaded as com-
pared to control (not loaded with Pi) microsomes (Fig. 1 A).

Similar experiments were run in microsomes pre-loaded using a freezing/thawing protocol. To this end, microsomal vesicles were opened/resealed in the presence or in the absence of 5 mM Pi. This manoeuvre increased the microsomal Pi loading up to 12.7 nmol per mg of protein, which should correspond to an intravesicular Pi concentration of 4.1 mM. Despite the increased gradient 
between intravesicular Pi and extravesicular G6P concentrations, the uptake of G6P was almost identical in Pi loaded and control microsomes (Fig. 1 B). In the experiments of both Fig. 1A and B, the prototypic G6PT inhibitor, S3483 [13], almost completely inhibited the G6P uptake.
Once in the (liver) ER/microsomal lumen, G6P is the substrate of at least two enzymes: G6Pase, and H6PD\[1–2,4\]. In case of G6Pase, the exit rate from microsomal vesicles of the reaction product glucose is lower than the G6Pase-mediated hydrolysis rate\[1,2,7\]. The product of H6PD, 6-phosphogluconate also accumulates within the microsomes \[4\]. Thus, upon the addition of \([\text{14C(U)}]\)G6P, the luminal presence of radiolabelled G6P, glucose and 6-phosphogluconate should be considered. To evaluate the net \([\text{14C(U)}]\)G6P uptake, the contribution of the intravesicular \([\text{14C(U)}]\)glucose produced by G6Pase activity to the total intravesicular \([\text{14C}]\) radioactivity was abolished by inhibiting the enzyme with 100\(\mu\text{M}\) vanadate \[6–8\]. Moreover, freezing/thawing of microsomes abolished H6PD activity in the microsomal lumen (see below). 100\(\mu\text{M}\) vanadate resulted in the maximal inhibition of G6Pase activity (\(94.3\%\), measured in ancillary experiments). As demonstrated in Fig. 1 C, the apparent uptake of labelled G6P was approximately fivefold lower as compared to microsomes without vanadate treatment (compare to Fig. 1 A and B), which is essentially in agreement with the direct measurement of intravesicular \([\text{14C(U)}]\)G6P and \([\text{14C(U)}]\)glucose content \[7\]. Under these experimental conditions too, there was no stimulatory effect by microsomal Pi pre-loading on G6P uptake (Fig. 1C). Both in Pi pre-loaded and control microsomes, the G6PT inhibitor S3483 largely blocked G6P uptake (Fig. 1C).

The uptake of a lower concentration of G6P (i.e. 50\(\mu\text{M}\)) was also evaluated (Fig. 2). Under these conditions, the ratio between the intravesicular concentration of Pi (in the millimolar range) and the extravesicular concentration of G6P (50\(\mu\text{M}\)) should even more favour a putative exchange between the two anions. However, either in the case of microsomal Pi loading by pre-incubation with Pi (Fig. 2A) or by the freezing/thawing procedure (Fig. 2B) the uptake of radioactivity was almost identical in Pi pre-loaded and in control microsomes. Again, the uptake was blocked by the G6PT inhibitor S3483 (Fig. 2A). When microsomal vesicles have not been opened/resealed (Fig. 2A) the apparent uptake of \([\text{14C(U)}]\)G6P was much over the passive equilibrium of G6P (approximately 4.5-folds). This can be explained by the intraluminal storage of \([\text{14C}]\)6-phosphogluconic acid due to the H6PD activity \[8,14\]. In fact, opening/resealing of the microsomal vesicles resulted in a much lower \([\text{14C(U)}]\)G6P uptake (Fig. 2B). In this latter case, the

Fig. 1. Pi pre-loading of liver microsomal vesicles does not affect the uptake of 1 mM G6P. Microsomes were pre-loaded by incubation in the presence of 5 mM Pi (panel A) or by a freezing/thawing procedure (panel B), as detailed in “Section 2”. G6P uptake was started upon dilution in a Pi-free medium containing 1 mM G6P plus trace amounts of \([\text{14C(U)}]\)G6P. Where indicated in figure, 30\(\mu\text{M}\) S3483 was added to the media for the Pi loading procedures, as well as in the dilution medium. G6P uptake was measured by a rapid filtration assay as detailed in “Section 2”. Data are means S.D. of three independent experiments.

Fig. 2. Pi pre-loading of liver microsomal vesicles does not affect the uptake of 50\(\mu\text{M}\) G6P. Microsomes were pre-loaded by incubation in the presence of 5 mM Pi (panel A) or by a freezing/thawing procedure (panel B), as detailed in “Section 2”. G6P uptake was started upon dilution in a Pi-free medium containing 50\(\mu\text{M}\) G6P plus trace amounts of \([\text{14C(U)}]\)G6P. Other experimental conditions are as reported in the legend to Fig. 1. Data are means S.D. of three independent experiments.
release of the intravesicular pyridine nucleotides and their very large dilution in the extravesicular medium, virtually abolished H6PD activity [14], thus reducing the apparent uptake of [14C(U)]G6P.

3.2. Microsomal G6P permeability evaluated by a light-scattering technique

As previously reported [6 and Refs. therein, 7], the permeability of microsomal vesicles towards different solutes can be estimated by a light-scattering technique. The assay was run after opening/resealing of the microsomal vesicles in order to release eventual intraluminal Pi and to avoid possible anion exchange. No detectable Pi anions were measured in the acid extract of these microsomes.

The addition of either 10 mM (Fig. 3A) or 5 mM G6P (Fig. 3B) caused a rapid shrinkage of microsomes (increase in light-scattering) followed by a swelling phase (decrease in light-scattering), which reflects G6P influx rate. Noticeably, the swelling phase was almost completely abolished by the G6PT inhibitor S3483, at both the G6P concentrations (Fig. 3A and B). The treatment of microsomes with 100 µM vanadate somewhat reduced the microsomal permeability to G6P; at 5 mM G6P the effect of vanadate was somehow evident but not statistically significant (Fig. 3B), at 10 mM G6P this effect was more evident and statistically significant (Fig. 3A).

3.3. Release and uptake of 32Pi in microsomes pre-loaded with Pi

Previous data in proteoliposomes containing the reconstituted G6PT protein indicate that the transporter can also act as a Pi–Pi antiporter, and that the G6P–Pi and Pi–Pi antiporter activity can be observed in both directions [9,10]. To ascertain these points in the liver microsomal system, (i) microsomes were pre-loaded with 5 mM Pi, plus trace amounts of 32Pi and the Pi efflux was monitored upon dilution with or without cold 1 mM G6P (Fig. 4A) or 5 mM Pi (Fig. 4B), and (ii) the influx of Pi (5 mM, plus 32Pi) was evaluated in microsomes preloaded with 5 mM cold Pi (Fig. 4C).

As shown in Fig. 4A, no increase in the rate of spontaneous Pi release was induced by G6P. Pi release and uptake rate were not affected by the presence of an almost equal concentration of the anion either at the inner or at outer side of the microsomal membrane (Fig. 4B and 4C).

3.4. Microsomal Pi permeability evaluated by a light-scattering technique

The microsomal permeability to Pi (5 mM) was also investigated by light-scattering. As shown in Fig. 4D, the microsomal vesicles appeared to be fairly permeable to Pi, but with lower rate of entry (t½ = 24.43 s) as compared to that observed with same concentration of G6P (i.e. 2.43 s, Fig. 4B). Vanadate (100 µM) did not affect the microsomal Pi permeability (not shown).

4. Discussion

The present results strongly suggest that, in native liver microsomal vesicles, G6PT operates as a facilitative uniporter. Importantly, the present results militate against the possibility that G6PT operates as either a G6P–Pi or a Pi–Pi antiport. Actually, pre-loading microsomal vesicles with Pi did not increase the uptake of both G6P and Pi as evaluated radioisotopically. Also, Pi release from Pi pre-loaded microsomal vesicles was not enhanced in media containing G6P or Pi.
The detected microsomal uptake of $^{14}$C-labelled G6P not only reflects the net G6P uptake – up to the equilibrium between the intra and extra vesicular fluids – but also the intravesicular entrapment of G6P-derived $^{14}$C-labelled metabolites, e.g. glucose and phosphogluconic acid (1–3, 4, 7). The net uptake of G6P was measured upon the almost complete inhibition of the G6Pase activity with vanadate and the elimination of H6PD activity by freezing and thawing. Even under these conditions, G6P uptake was not enhanced at all by microsomal Pi pre-loading. Consistently, light-scattering measurements revealed a high permeability of microsomal vesicles for G6P, which do not contain any Pi inside, even when the G6Pase-mediated intraluminal Pi generation was suppressed by vanadate. These observations also indicate that the possibility for an exchange between Pi generated by the G6Pase activity within the vesicle lumen and the extra vesicular G6P is unlikely.

A high permeability of liver microsomes for G6P has been previously described in light-scattering assays and assumed to reflect G6PT activity because of its high specificity [e.g., 6]. Here we add an important piece of evidence to this interpretation since the G6PT inhibitor S3483 blocked the G6P influx into microsomal vesicles. Light-scattering measurements also confirmed that Pi could enter microsomal vesicles independently of intraluminal Pi content.

Previous experimental data are consistent with the present ones. It was shown that intravesicular G6P formed by phosphotransferase activity of G6Pase [15], leaves microsomal lumen through an S3483-inhibitable transporter, allegedly G6PT [4]; in this case, both G6P and Pi exit from the vesicles in the same direction. Moreover, treating liver microsomes with PbCl$_2$ resulted in the intravesicular entrapping of Pi derived by G6P hydrolysis as Pb–Pi complexes, which markedly reduced Pi efflux [8]; nonetheless, the rate of G6P hydrolysis – reflecting the rate of G6P influx – was not modified [4].

At variance with the present results, G6P–Pi and Pi–Pi antiport activities have been observed in proteoliposomes including the reconstituted G6PT protein [9,10]. An additional discrepancy between those and the present study are that vanadate was not inhibitory on the transport in our experiments. Because of the results shown in Figs. 1 and 3 we can conclude that vanadate – at least at 100 μM concentration, sufficient to inhibit G6Pase activity – does not block G6P transport. Instead, vanadate by inhibiting microsomal G6Pase activity does not allow the intravesicular accumulation of radiolabeled glucose derived from radiolabeled G6P, which results in a virtual lower uptake. The fact that an apparent (and any way minor) vanadate inhibition of microsomal G6P permeability was observed in light-scattering measurements (Fig. 3) can be logically explained by the lack of intra vesicular formation.

Fig. 4. Release and uptake of $^{32}$Pi in microsomes pre-loaded with Pi, and Pi influx into liver microsomal vesicles evaluated by light-scattering. Microsomes were pre-loaded with 5 mM Pi plus trace amounts of $^{32}$Pi by a freezing/thawing procedure, and $^{32}$Pi release was started upon dilution in a Pi-free medium containing 1 mM G6P (panel A) or 5 mM Pi (panel B). In panel C, microsomes were pre-loaded with 5 mM cold Pi by a freezing/thawing procedure and Pi uptake was started upon dilution in a medium containing 5 mM Pi plus trace amounts of $^{32}$Pi. Microsomal Pi content was measured by a rapid filtration assay as detailed in “Section 2”. Data are means (S.D. of three independent experiments. Panel D: the experimental conditions for the light-scattering measurements and the calculation of $t_{1/2}$ influx rate are those reported in the legend to Fig. 3. Where indicated by the arrow, a small volume of 0.25 M Pi (as K-Pi buffer, pH 7) was added to incubates to have a final concentration of 5 mM Pi. The light-scattering signal was acquired each 0.2 s in individual traces of four separate experiments, and the trace shown is the mean of the individual traces. The mean ± SD values of $t_{1/2}$ of influx rate – calculated from the individual traces – was 24.43 ± 5.0 s.
of glucose and Pi; those compounds can indeed contribute to the acceleration of the swelling phase in control microsomes.

The fact that G6PT operates as a G6P–Pi antiport once reconstituted in proteoliposomes can be theoretically explained in several ways. The transporter protein might be folded and oriented, either in the host cell ER membrane (COS-1 cells) or (and) in the reconstituted proteoliposomal membrane, differently from the native environment, i.e. the liver ER membrane. Also, the high content in the overexpressed reconstituted G6PT versus the content of other transporters (e.g. that for Pi, K⁺, Na⁺) of the microsomes used for the proteoliposomal assembly can somehow favour exchange reactions. It should be noted that for the G6P–Pi exchange the stoichiometry was far from the equilibrium: 0.2 mM for the former versus 50 mM for the latter [9,10]. Moreover, the high concentrations of external Pi/G6P applied – as competitive inhibitors – might also osmotically influence the internal volume of the proteoliposomes, modifying the rate of the uptake.

In any event, our findings show that the G6PT works as a G6P uniporter under circumstances resembling the (liver) physiological conditions. In this respect, we should consider that cytosolic inorganic Pi does not exceed a 5 mM concentration [16]. It can be argued that higher Pi concentrations are present in the ER lumen, particularly at sites neighbouring the G6Pase enzyme. This argumentation is, however, untenable since G6Pase activity does not appear to be required for G6PT-mediated transport of G6P. Consistently with this conclusion, no close vicinity between G6PT and G6Pase proteins has been demonstrated, as a lack of FRET was observed between the two fluorescent proteins [17]. Under physiological conditions another point deserves attention: G6Pase activity is not the only source of intrareticular Pi, since ER Pi content can vary depending on ER Ca²⁺ storage [18 and Refs. therein], glucuronidation [2], ATP hydrolysis [2] and, possibly, other dephosphorylation reactions. This would imply, according to the G6P–Pi exchange hypothesis, undesired interference with the rate of G6P hydrolysis. Moreover, assuming that the exchange reaction is the sole pathway for Pi efflux from ER to cytosol, additional severe defects should be present in patients affected by the inherited deficiency of G6PT, i.e. in glycogen storage disease type 1 non-a. The observation that all the patients affected by glycogen storage disease type 1 non-a. Eur. J. Hum. Gen. 7, 717–723.

Collectively, the present results compellingly indicate that, at least in the major glucogenic tissue, the liver, G6PT acts as a facilitative uniporter. This conclusion appears consistent with several physiological considerations, but does not exclude that other gene products related to G6PT – i.e. the SLC37A1 and SLC37A2 products, poorly expressed in the liver [9] – may operate as G6P–Pi exchangers, particularly in non glucogenic tissues/cells. This latter point would deserve further investigation.

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